IMPROVEMENTS IN IN VITRO REARING METHODS OF *TOXONEURON NIGRICEPS* (VIERECK) (HYMEOPTERA: BRACONIDAE), A LARVAL ENDOPARASITOID OF *HELIOTHIS VIRESCENS* (LEPIDOPTERA: NOCTUIDAE)

A Dissertation

by

INDIRA KURIACHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Entomology

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Approved as to style and content by:

S. Bradleigh Vinson (Chair of Committee)	Roger E. Gold (Member)
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ABSTRACT

Improvements in in vitro Rearing Methods of *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae), a Larval Endoparasitoid of *Heliothis virescens* (Lepidoptera: Noctuidae). (May 2005)

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Chair of Advisory Committee: Dr. S. Bradleigh Vinson

This project focused on improving the in vitro rearing methods of *Toxoneuron nigriceps*, an important biological control agent of *Heliothis virescens*, by supplementing the rearing media with the host hemolymph. *T. nigriceps* larvae of different ages were incubated in the artificial rearing media. The growth (increase in length and width), development (molting), and survival of the incubated larvae were observed. Changes in osmotic pressure of the rearing media before and after incubation were evaluated. The protein profiles of day 1, 3 and 5 hemolymph samples of the parasitized and unparasitized *H. virescens* fifth instar larvae, used in the artificial rearing media, were determined.

Host hemolymph improved the growth and development of *T. nigriceps* larvae in the artificial rearing media except in the case of new and early first instar larvae. Osmotic pressure of all media showed a significant decrease after the incubation of the larvae except media incubated with the new first instar larvae indicating that the older

larvae absorbed and utilized more of the nutrients from the rearing media than the younger larvae. Growth and development was higher in the semisolid media than in the liquid media. In the artificial rearing media, the late first instar *T. nigriceps* larvae molted to second instars and a few of them molted to third instars. The early and late second instar larvae incubated in the artificial media also grew well and molted to third instars. Some of the in vitro reared third instar larvae demonstrated behavioral changes that could be expressed as the preparation for cocoon formation or pupation, however neither a cocoon nor pupation occurred.

No qualitative differences in the protein titers were detected between hemolymph of the parasitized and unparasitized fifth instar *H. virescens* larvae. There was a significant increase in the protein concentration in both unparasitized and parasitized *H. virescens* larvae as the age increased. This increase in the protein concentration showed a positive effect on the growth and development of *T. nigriceps* larvae indicating that availability of nutrients was an important factor for the growth of *T. nigriceps* larvae in vitro.

This dissertation is dedicated to:

All women who dare to return to school after a long break and to continue their education while fulfilling their responsibilities as a wife and a mother.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. S. Bradleigh Vinson for giving me the opportunity to pursue my Ph.D. while working full time, and for his guidance and support throughout the course of my study. I am grateful to Dr. Gold, my committee member, for his invaluable advice and encouragement which helped me to reach this point. I wish to thank the other members of my advisory committee, Dr. Howard Williams and Dr. Larry Johnson for their support and interest in my project.

I want to express my deepest gratitude to Dr. Fernando Consoli who was like a mentor to me. I really appreciate his generosity and patience to help me with all the problems with the methodology during the initial stages of this project. He was always available to discuss the problems and give me valuable suggestions.

My sincere appreciation to Dr. Asha Rao for helping me with the statistical analysis. Special thanks to Ms. Sherry Ellison, my colleague who was always been there for me to share my ups and downs on this project and give me some magic tips to get over the blues. I want to share my gratitude with my student workers Larry, Dustin, and Elizabeth for helping in the rearing and maintenance of the moths and the parasitoid wasps.

I wish to acknowledge Dr. Larry Dangott and the Protein Chemistry Laboratory crew for conducting the 2D gel electrophoresis.

From the depth of my heart, I wish to thank my husband, Adattu Kuriachan, my two sons, Vikas and Vipin, my daughter in law, Anu and my special mother,

Mrs. Subadra Nair, for their continued love, support, patience and encouragement throughout my academic path.

Finally, I wish to thank my ex-boss, my friends, and relatives who were always with me to boost my confidence and keep me in good spirits that helped me reach where I am today.

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I INTRODUCTION

The parasitic Hymenoptera are a group of insects which contains many thousands of species that parasitize insects and other arthropods, and are collectively known as parasitoid wasps (Godfray, 1994). The adult female wasps lay their eggs either in or on the host insect. From that point onwards, the parasitoids are constrained to complete their larval development in or on a single host, often in or on a single host developmental stage. Insect parasitoids are carnivores that develop at the expense of their host, which ultimately dies having failed to reproduce (Vinson et al., 2001). Parasitoids are organisms that are free living as adults, but their young are dependent on the adult females selecting a single host from which immature stages must derive all of their nutrition. There is no opportunity for immature parasitoids to chose or move to a different resource. The immature parasitoid stages are initially parasitic, but later in their development often act more like predators, although they only consume one prey item- the host (Vinson et al., 2001).

Although most insect orders are attacked by parasitoids, the holometabulous orders appear to be attacked by a number of different parasitoids. The range of holometabulous hosts utilized by hymenopteran parasitoids extends to several insect orders including Coleoptera, Diptera, and even other Hymenoptera; but these parasitoids display ‘an inordinate fondness’ for the larval stages of the Lepidoptera which comprises

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The style and format of this dissertation follow the Journal of Insect Physiology.

the butterflies and moths (Edwards et al., 2001). This 'inordinate fondness' for the larval stages of the butterflies and moths makes the parasitoid wasps, a potential candidate for the biological control of these pest insects.

Due to increased concern over the use of pesticides, biological control has become one of the more important components of integrated pest management programs (IPM). However, biological control has relied on conservation and importation of natural enemies. Though, many imported species are effective, they do not establish. Another approach is rearing and release known as augmentation. The use of parasitoids in augmentation research and in large-scale biocontrol programs is limited by labor costs, technical problems, and expenses associated with rearing both a host and a parasitoid when only the parasitoid is needed (Nettles 1990). In vitro rearing of insect parasitoids is one possible way to solve this problem (Nettles, 1990, Greany, 1986, Vinson, 1994). Besides, the use of in vitro culture systems has received a great deal of attention in order to elucidate complex physiological interactions between larval endoparasitoids and their hosts (Grenier et al., 1994, Vinson 1994, Vinson and Hegasi, 1998), because parasitoid larvae can be directly observed and examined without having to cut open the host.

Prell (1915) carried out the earliest known trial to rear a larval endoparasitoid. He tried to rear a parasitic tachinid, *Parasitigen sitvestres* in chicken albumin. After that, numerous attempts have been made to raise parasitoids in artificial diet. The first attempts to develop parasitoids on artificial diets focused on the life history of the immature stages. Afterwards, the studies were primarily designed to investigate the physiology and the nutritional requirements of parasitic insects (Consoli and Parra,

1999). House (1954) was the first to rear the parasitic sarcophagid P*seudosarcophaga affinis* successfully on a chemically defined diet. Thus, successes in the in vitro rearing of parasitoids began in the 1950s and today more than 70 species of parasitoids are partially or completely reared on artificial diets (Consoli and Parra, 1999). Progress has been made in the development of artificial media for the in vitro rearing of ectoparasitoids, egg, and pupal endoparasitoids, as more than 25 species of the stated parasitoids have been reared on artificial diets from egg to adult (Hu and Vinson, 1998, Vinson, 1994, Grenier, 1994). However, the successful in vitro rearing of hymenopteran larval endoparasitoids from egg to adult has not been reported (Hu & Vinson, 1998). A few attempts that were reported to be partly successful including: a) *Microplitis croceipis* (Hymenoptera: Braconidae) which have only been reared from egg to first instar (Greany 1986, Ferkovisch et al., 1990); b) *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) from egg to prepupa (Hu and Vinson, 1998); c) *Toxoneuron nigriceps* (Hymenoptera: Braconidae) from egg to second instar (Pennacchio et al., 1992); and, d) *Lysiphlebus fabarum* (Hymenoptera: Braconidae) from a first instar larva to adult (Rotundo et al., 1988).

Although some parasitoid species have been reared on a diet devoid of insect material in vitro, many others resulted in poor growth and development in the absence of host components in the artificial rearing media. Only 14 species of parasitoid had been reared to the adult stage in the absence of host components and these include sarcophagids (five species), icheneumonids (three species), tachinids (three species), and a single trichogrammatid species. For the tachinid and trichogrammatid species reared to

the adult stage on artificial diet without host components, yields and parasitoid quality were low. *In vitro* rearing of *Muscidifurax zaraptor*, a pupal parsitoid wasp of the muscoid flies, resulted in a higher yield of adult wasps in artificial diet with insect material as compared to the yield in the diet without insect material. Furthermore, the parasitoid quality was superior for insects fed diet with insect material compared to those fed diet devoid of insect material (Fanti and Vinson, 2000). Nettles (1990) suggested that host metabolites (host factors) are likely to be necessary components for successful *in vitro* rearing of several parasitoid species. This project focused on improving the artificial rearing techniques of *Toxoneuron nigriceps,* formerly known as *Cardiochiles nigriceps,* a larval endoparasitoid of *Heliothis virescens* by the addition of host hemolymph proteins.

The host

Heliothis virescens, the tobacco budworm, is an economically important pest insect that attacks tobacco, cotton and vegetables including tomatoes and garden peas (Davidson and Lyon, 1979, Huffaker, 1985, Metcalf and Metcalf, 1993). It is a native species found throughout eastern and southwestern United States and in California. The tobacco budworm overwinters in the soil as pupa; the moth emerges in the spring. Females lay single eggs on the underside of the leaves in five days and development is completed in 18-31 days (Davidson and Lyon, 1979, Metcalf and Metcalf, 1993). Females normally produce 300-500 eggs, but 1000 to 1500 eggs per female have been reported from larvae cultured on artificial diet (Fye and McAda, 1972). Tobacco budworm larvae have five to seven instars, with five or six most common. The

developmental time from hatching to pupation is approximately 10-12 days. The larvae of *H. virescens* eat into the buds and unfolded leaves of both cotton and tobacco, which reduces quantity and quality of these crops (Huffaker, 1985; Metcalf and Metcalf, 1993). Developing insecticide resistance in this pest has resulted in an effort to use biological control agents (Barras, 1970, Huffaker, 1985). Parasitism by *Toxoneuron nigriceps* may serve as one element in an integrated management program designed to minimize the use of chemical control and reduce the population of this pest. However, the number of adult parasitoid wasps is low in the season when the host population is high due to the low level of parasitism. Therefore, augmentative release of adult wasps needs to be considered in the control of the tobacco budworm.

The parasitoid

Toxoneuron nigriceps is a solitary larval endoparasitoid of tobacco budworm and related species of *Heliothis*. Although most species of parasitic wasps are small and inconspicuous, this is not true of *T. nigriceps*. The adult wasp is larger than most other parasitic wasps, approximately 7 mm in length, with black wings and a red abdomen. Tobacco budworm and related species of *Heliothis* are the primary hosts of this parasite. This parasitic wasp is found to be an important biological control agent of the tobacco budworm. A two -year study on the population density of this wasp and corresponding parasitism of *Heliothis* larvae on cotton indicated as more than 80% parasitism at desities of 400-600 *T. nigriceps* females per acre (Lewis et al., 1972). The parasitized caterpillar larvae grow slower and cause less damage than normal. Adult wasps will "sting" or lay eggs in tobacco budworm larvae of all sizes, but they prefer younger

larvae. Older larvae are better able to 'fight off' the wasp and keep her from successfully laying eggs. The translucent white egg is inserted into the body cavity of the host. The egg hatches 32 to 48 hours after oviposition (Lewis and Vinson, 1968); however, in the laboratory, the eggs hatch between 32 to 36 hours at 29± 1°C (personal observation).

Immediately following hatching, the first instar larvae average 0.67mm long and 0.236 mm across the head. The newly molted second instar larvae average 3.67 mm long, with the average greatest width of 0.90mm. The newly molted third instar larvae average 9.00mm long and have an average greatest width of 2.6mm (Lewis and Vinson, 1968). The wasp larva hatches inside the caterpillar and begins feeding on the hemolymph of the host. However, its growth is slow and it remains as a first instar larvae until the budworm larva (host) enters the fifth instar which is close to pupation. Then the wasp larva grows rapidly until it becomes the third instar.

The third instar larva emerges head first from the metathoracic region of the host. When the parasitoid larva is almost free of the host larva, but is still attached by its posterior caudal horn, it reinserts its head and begins feeding at the posterior end of the host remains. As the larva feeds, it works toward the anterior end of the host remains, and completely consumes all remaining body fluids and tissues, leaving only the head capsule and the integument. Shortly after complete emergence, the larva spins a white silken cocoon. The developmental time is 5-6 days for the first instar, 2-3 days for the second instar, and 2 days for the third instar (Lewis and Vinson 1968). Normally, adult wasps emerge from the cocoons within a week. In adverse conditions, the wasps diapause at pupal stage. The male wasps emerge first. Adult wasps live for about 3-4

weeks. But in the laboratory, the female wasps are alive for 5-6 weeks (personal observation).

Physiological association between the larval endoparasitoids and their hosts

The great complexity of relationships between the endoparasitoids and their hosts is thought to create a difficult challenge for the development of methods to rear these parasitoids in in vitro (Vinson, 1994*)*. These complex relationships involve physiological and biochemical interactions between the developing endoparasitoid and its host which result in a change in requisite needs as development proceeds (Vinson, 1988). These changes include the immune system, nutritional, respiratory, excretory, and endocrinological considerations, as well as changing physical environmental conditions such as humidity, surrounding substrate consistency, and luminosity (Grenier et al., 1994). The parasitoids alter the physiology of the host in favor of their own growth and development by injecting chemicals like venoms, calyx fluid, polydnavirus and teratocytes, along with the egg. These chemicals disrupt the host immune system, suppress protein synthesis and inhibit the host pupation by blocking ecdysone synthesis from the prothoracic gland (Vinson, 1972, Guillot and Vinson, 1972). This altered host physiology may affect the nutrition of the parasitoid. An understanding of how the nutritional ecology of the multitrophic interactions involving the parasitoid, the host, and the food of the host may affect the development of the parasitoid, is necessary for the development of nutritionally adequate artificial media (Consoli and Parra, 1999).

For some species that parasitize lepidopteran or dipteran hosts, it has been recognized that development of the parasitoid was arrested at the first instar stage in the

immature host, and then the growth and development of the parasitoid resumed when the host reached the last instar or pupal stages (Pennacchio et al., 1993). This pattern of temporary developmental arrest and resumption of growth may be related to the changes in the concentration of the hemolymph proteins of the host. The concentration of these proteins, collectively called storage proteins, increases markedly in the hemolymph of the final instar larvae of holometabolous insects comprising more than 80% of the total hemolymph protein by weight (Leclerc and Miller, 1990). The storage proteins have a high content of aromatic amino acids, which consists of up to 25% phenylalanine and tyrosine (Wyatt and Pan, 1978). Aromatic amino acids are essential to the extensive exoskeleton formation accompanying metamorphosis (Kramer et al., 1980).

The larval development of *Toxoneuron nigriceps* Viereck, a solitary larval endoparasitoid of *Heliothis virescens* (F), the tobacco budworm, occurs in the last instar larva of the host (Pennacchio et al., 1993). Regardless of what stage of the host was parasitized, the parasitoid remained as a first instar larva until the host molted to the fifth instar. In both parasitized and unparasitized *H. virescens*, the protein concentration of the hemolymph rose sharply on day 2 of the 5^{th} larval instar. In unparasitized larvae, there was a decrease in the hemolymph protein concentration beginning on day 5, whereas the parasitized larvae showed a continuous increase in the protein concentration of the hemolymph (Pennacchio et al., 1993). This allowed the parasitoid to exploit the total nutritional increase, occurring in the host, in preparation for metamorphosis. The host ligation experiments conducted by Pennacchio et al. (1993) supported this view. The authors found that ligations performed before day 2 of the 5^{th} instar host completely

inhibited larval development of T. *nigriceps*; however, later ligations did not apparently interfere with parasitoid development.

Nutritional requirements of endoparasitoids

Parasitoids share the same qualitative nutritional requirements of other insects which include proteins, carbohydrates, lipids, sterols, vitamins and salts. However, the protein content should be high unlike the phytophagous insects which may require different ratios of carbohydrates and proteins for their development (Thompson 1999, House, 1961). Yet, the fact that the parasitoids live in an ongoing and changing habitat (host), to identify the changing nutritional needs of the parasitoid becomes a challenge. House (1977) suggested that parasitoids have essentially the same nutritional requirements as other insects. The 10 essential amino acids are usually required and the other 10 non-essential amino acids are beneficial for normal growth of some parasitoids (Nettles et al., 1980). Additionally, Nettles (1990) also found that certain chemicals present in the host may have become essential dietary requisites for some parasitoids, as a result of evolutionary changes. Proteins were also essential as a nitrogen source to provide amino acids without raising the osmotic pressure of the artificial diets (Pennacchio et al., 1992, Greany, 1986, Thompson, 1980, 1983, and 1986), except in some parasitoids that lack proteinase (Nettles, 1990). While certain specific nutrients may occasionally be in short supply in the host, it is the amino acids and proteins that are most likely to be limiting to the growth of the parasitoid. In addition, the specific amino acid composition of the host proteins may be particularly important (Vinson et al., 2001). In chordates, there is a dietary need for calcium as a major component of the

skeleton. By contrast, in insect lines, there is a reduced need for calcium (Taylor, 1986), but an increased dietary requirement for the components of the cuticle such as chitin and proteins. For example, both tyrosine and β-alanine appear to play an important role in cuticle sclerotization. (Andersen, 1985, Chen, 1985, Sherald, 1980). Since tyrosine has low solubility, it becomes more accessible as a resource when present as a constituent of a protein. In addition, most of these amino acids are locked-up in the cuticle of the host and are therefore unavailable to the developing parasitoid until the hosts molt or until the latter stages of host development when storage proteins appear in the hemolymph (Vinson et al., 2001). The composition of the proteins that are available to the developing parasitoid may change as the host develops, and the nutritional needs of the parasitoid may also change, as it develops. Thus, in vivo, the parasitoids live inside the host hemolymph which presents a continuously changing environment that creates a changing, but adequate supply of the nutritional needs for the developing parasitoid.

Hemolymph proteins

Insect hemolymph contains many different proteins with a variety of functions. The total quantity of protein in the hemolymph varies over the course of development, but peak concentrations are found in the late larval stages of development. These proteins include storage proteins, lipid transport proteins, vitellogenins, enzymes, proteinase inhibitors, chromoproteins, and a range of different proteins that are involved in the immune responses of insects (Chapman, 1998). The storage proteins are generally synthesized in the fat body of insects in their last larval stage, and secreted into the hemolymph. Shortly before pupation, storage proteins are taken up by fat body tissue

and stored there, presumably as protein granules, until their final utilization for the biosynthesis of adult structures (Wang and Haunerland, 1991). The term 'storage proteins' is generally suitable to define any of the 500-kDa, abundant larval hemolymph proteins that achieve high concentrations during larval life and diminish during adult development (Kanost et al., 1990). Storage proteins have been primarily studied in Lepidoptera and Diptera, but are widespread in their occurrence across the insect orders. Storage proteins are hexamerins, *i.e*. have six subunits; the subunits may all be the same, or may be of two or three different types (Chapman, 1998). Most insects studied have only one or two different storage proteins, but some Lepidoptera have three or four (Chapman, 1998). One of the main classes of storage proteins in Lepidoptera is the Arylphorins which have a high content of aromatic amino acids (phenylalanine and tyrosine comprise 18-26% of the total) and a low content of methionine (Chapman, 1998). Similar proteins occur in Coleoptera, Diptera and Hymenoptera, but the proportion of aromatic amino acids is less (about 12%). Arylphorins are degraded in the developing adult either in the fat body or their tissue, and are used to supply necessary amino acids for remodeling of tissues and sclerotization of cuticle (Karpells et al., 1990). Lepidoptera also has a second class of storage protein designated as methionine-rich protein that contains 4-8% methionine. In Hymenoptera, the second protein has high levels of glutamine/glutamic acid (Wheeler and Buck, 1995). In the tobacco budworm, three abundant storage proteins have been detected in the fifth instar larval and pupal hemolymph and pupal fat body (Leclerc and Miller, 1990). These polypeptides have subunit molecular weights of 74,000; 76,000; and 82,000-M_r (designated p74, p76 and

p82, respectively) as determined by SDS-PAGE, and exist as 450,000-M_r hexamers in their native state (Leclerc and Miller, 1990). These storage proteins are absorbed by the fat body after the 5th day of fifth instar *Heliothis* larvae.

Changes in hemolymph protein titer caused by parasitization

Studies have shown that parasitization results in a significant change in the protein titer of the host. Rahbe et al. (2002) reported a four-fold increase in tyrosine accumulation in the hemolymph of the pea aphid, *Acyrthosiphon pisum* (Homoptera: Aphididae), when parasitized by *Aphidius ervi* Haliday (Hymenoptera: Braconidae) as compared to the non-parasitized aphid. A significant decrease in arylphorin titer was reported in the hemolymph of *H. virescens* larvae parasitized by *Microplitis croceipes* (Dong et al., 1996). Similarly, there was a reduction in the protein titer of the hemolymph of the tobacco budworm larvae parasitized *by Campoletis sonorensis* (Hu, 1996). *H. veriscens* larvae parasitized by *T. nigriceps*, showed no significant differences in hemolymph protein concentration from unparasitized controls up to the peak occurrence on day 4 of the host fifth instar. Beginning on day 5 of the parasitized fifth instar, instead of decreasing as in unparasitized larvae, the protein concentration of the hemolymph increased and was significantly greater than in unparasitized controls. The highest hemolymph protein value was registered on day 7 of the 5th instar of parasitized larvae (Pennacchio et al., 1993).

I wanted to investigate whether the presence of host hemolymph proteins in the artificial rearing media would promote the growth and development of *T. nigriceps*. I hypothesized that there would not be any difference in the growth and development of

the parasitoid larva in artificial rearing media with hemolymph from both parasitized and unparastitized *H. virescens* larvae on day 1, 3 and 5 of the fifth instar. Furthermore, I wanted to compare the protein profile of the hemolymph of the parasitized and unparasitized *H. virescens* fifth instar larvae, in case my hypothesis failed.

I selected 2D Gel Electrophoresis to separate and identify the hemolymph proteins of these insects. 2D Gel Electrophoresis is a method for the separation and identification of proteins in a sample by displacement in 2 dimensions oriented at right angles to one another. This allows the sample to separate over a larger area, increasing the resolution of each component (Gorg et al., 2000). This method is generally used as a component of proteomics and is the step used for the isolation of proteins for further characterization by mass spectrometry. Other main purposes for using this method are for the large scale identification of all proteins in a sample and to compare two or more samples to find differences in their protein expression. However, 2D gel electrophoresis has a few disadvantages as any other technique. The proteins that are more than 100kD may not be visible in a 2D gel. Besides, there can be more than one protein in one spot which may appear like a very dark spot. 2D gel electrphoresis is performed at two steps – isoelectric focusing (IEF) is used in the Ist dimension (Righetti, P.G., 1983). This separates proteins by their charge (pI). SDS-PAGE is used in the 2nd dimension. This separates proteins by their size (molecular weight).

For my dissertation project, I investigated the growth and development of the red-tailed wasp, *Toxoneuron nigriceps,* in a semisolid and a liquid artificial rearing media with the addition of the host hemolymph. An artificial basic medium was

developed by modifying the Hu diet (Hu and Vinson, 1997) to which the hemolymph of the host larvae was added.

General objective

The main objective of this project was to improve the in vitro rearing technique for the braconid *Toxoneuron nigriceps*, a solitary larval parasitoid of the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), and to determine if host factors (hemolymph proteins) or parasitoid directed host factors were important in an artificial rearing medium.

Specific objectives

1. To develop an artificial basic medium (ABM) comprised of the essential amino acids and proteins.
2. a. To evaluate the effect of hemolymph proteins, including the storage proteins extracted from the day 1, day 3, and day 5 of the fifth instar larvae of the host, *H. virescens* (both parasitized by *T. nigriceps* and unparasitized larvae), added to ABM in the growth and development of *T. nigriceps* larvae which were transplanted after 2 days, 4 days, 6 days, 8 days and 10 days from parasitization. From now on, these larvae will be referred to as new first instar (2 days after parasitization), early first instar (4 days after parasitization), late first instar (6 days after parasitization), early second instar (8 days after parasitization) and late second instar (10 days after parasitization).

b. To evaluate the suitability of a liquid and a semisolid artificial rearing media in the growth and development of the new first instar, early first instar, late first instar, early second instar, and late second instar larvae of *T. nigriceps*

c. To compare the osmotic pressure of each artificial rearing media before and after the incubation with the parasitoid larvae.

3. To compare the hemolymph protein profile: 1) between the day 1, day 3 and day 5 of the unparasitized fifth instar *H. virescens* larvae, 2) between the day 1, day 3 and day 5 of the fifth instar parasitized *H. virescens* larvae, and, 3) between day 1, day 3 and day 5 of the unparasitized and parasitized fifth instar *H. virescens* larvae.

II MATERIALS AND METHODS

Insect cultures and rearing

The host, *H. virescens,* was reared on Bio-Serv corn earworm diet using established methods (Raulston & King, 1984). The parasitoid, *T. nigriceps,* was reared on larvae of *H. virescens* in the laboratory (Vinson et al., 1973). The parasitoid adults were separated by sex, and were fed daily with a 40% honey/water solution. For mating, the females and male wasps were kept together in the ratio 2:1 for 24 hours in a cage and afterwards the mated females and the males were kept separately. The adult wasps were maintained at 25±1° C and at ambient RH (about 45-50%) with a 14 h light: 10h dark photoperiod. The parasitized and unparasitized *H. virescens* larvae were maintained in the incubator at 29 ± 1° C and 14h light : 10 h dark. The 4^{th} instar and the 5^{th} instars of *H.virescens* larvae were identified based on the head capsule width (Webb and Dahlman, 1985). At 24 h after ecdysis, the 4^{th} instar (prior to the head capsule slippage condition), *H. virescens* larvae were parasitized.

Parasitization of the *H. virescens* larvae

H. virescens larvae were individually parasitized to avoid superparasitization. To parasitize, the host larva was exposed to the wasp one at a time by placing the larvae and 2 mated female wasps together under a 30mmX 5mm Petri dish. As soon as the larvae was stung by one of the wasps one time, the larvae would be removed and another larvae was introduced. The same 2 wasps were used to parasitize a maximum of 10 larvae at a time. The parasitized host larva was placed individually in a plastic vial

containing a 12 mm X 12 mm piece of diet (Raulston & King, 1984) with the open end of the vial plugged with cotton.

Preparation of the artificial basic medium (ABM)

Based on previous studies on the artificial diets of the larval endoparasitoids of *H. virescens* (Pennacchio et al. 1992, Hu and Vinson, 1997) and preliminary trials, an artificial basic medium was prepared using TNM-FH insect medium. Approximately 50 ml of TNM-FH insect medium was taken in a 200 ml beaker which was continuously stirred at medium speed using a magnetic stirrer. To this medium, 20 ml of fetal bovine serum was added. Then, 1 g of each trehalose, lysine, asparagine, glutamine, hydroxyproline, serine, threonine, valine, bovine serum albumin and 0.5 g of lactalbumin were added one by one, respectively. Each component was completely dissolved before addition of the next. Later, the solution was brought up to 100 ml by adding additional TNM-FH medium. The medium was then sterilized by passing it through 0.22 μm filter (Millipore Corporation, Bedford, MA 01730). Antibiotic-Antimycotic (GIBCO, Grand Island, N.Y.14072) was added (2 ml of 2% solution) and the final solution was stored in the refrigerator.

Hemolymph collection from the parasitized and unparasitized *H. virescens* larvae to use in the artificial basic medium (ABM)

Hemolymph was collected from day 1, day 3, and day 5 of the fifth instar parasitized by *T. nigriceps* and unparasitized *H. virescens* larvae. An aliquot of 750 μl of anticoagulant buffer (Strand et al., 1997) was pipetted out to a 1.7ml centrifuge tube which was already placed on ice. The larva was washed with water and then the tip of a

proleg was cut with the microscissors and the extruding hemolymph was extracted directly into the buffer using a syringe and a needle. The hemolymph was pooled from several larvae of the same age to collect 500 µl in each 1.7ml centrifuge tube and the tube was placed on a water bath at 62°C for 2 min to destroy phenoloxidases. The heat-treated tube containing hemolymph was centrifuged at 500 g for 10 min and the supernatant was then concentrated using centrifugal filter device of 10000NMWL. The concentrated hemolymph was kept in the ultra-cold freezer at -80°C until it was used for the experiment.

Hemolymph collection from the parasitized and unparasitized *H. virescens* larvae to use in the 2D gel electrophoresis

Hemolymph from the day 1, day 3, and day 5 day of the fifth instar *H. virescens* larvae (both parasitized and unparasitized) was obtained by cutting the tip of the proleg and collecting in the TBS homogenization buffer (Martinez and Wheeler, 1991) with the protease inhibitor cocktail (Sigma) in the ratio 10µl hemolymph: 90µl buffer. The sample was then centrifuged at 500 g for 10 min and 90 µl of the supernatant was extracted to another centrifuge tube. From this 90 µl, 10 µl was transferred to another tube to be used for the protein assay.

Since the protein quantity was low in the one day old larvae, the hemolymph was pooled from several larvae to standardize the protein quantity with the 3 d and 5 d samples. But for the 3 d and 5 d old hemolymph, only single larvae were used.

Dissection and collection of *T. nigriceps* new first instar, early first instar, late first instar, new second instar, and late second instar larvae for the tests

The parasitized *H. veriscens* larvae were surface sterilized in 95% ethanol for 1 min and then in 70% ethanol for 10 min; thereafter the larvae were transferred to sterilized water. For dissection, the larva was placed in a 35X 10 mm Petri dish with 6 ml of TNM-FH (Sigma) insect medium. Using microscissors, the larva was cut along the dorsal mid line starting from the anterior end (from the 3rd segment after the head capsule) to the posterior end without damaging the gut. The body cavity of the host was flushed with the insect medium to liberate the parasitoid larvae which were then collected with a sterile pastuer pipet and placed into another 35 X 10mm petri dish containing TNM-FH medium. The larvae were rinsed three times with fresh TNM-FH medium before transferring to the artificial rearing medium.

Preparation of 2% agar

Distilled water (100ml) was placed in a sterilized bottle. Next, 2 g of agar was added and the agar-water mixture in the bottle was heated on a heater while being constantly stirred. The heater was tuned off when the agar was completely dissolved. After cooling, the agar was autoclaved for 20 min and stored at room temperature.

Collection of fresh chicken egg yolk

A chicken egg was first surface sterilized by placing it in a beaker containing 70% ethanol for 15 min. Then, the eggshell was removed from the top with sterile forceps; next, the egg white was removed using a 35 cc sterile syringe, and the egg yolk

was collected with a 1 cc sterile syringe (Hu and Vinson, 1997). The egg yolk was added to the media immediately to avoid yolk coagulation.

Preparation of the liquid and semisolid rearing medium

Hemolymph 50%, 40% of the ABM, 10% (v/v) fresh chicken egg yolk and 0.001% (w/v) of 20- hydroxyecdysone (Sigma) were mixed together. The hemolymph was sterilized by passing it through 0.22 μm filter before adding to the media. Finally, 2% of Antibiotic-Antimycotic (Sigma) was added. Depending on the age of the hemolymph added, the different media were named as UP1, UP3, UP5, P1, P3, and P5 respectively. For example, UP1 stands for the media containing artificial basic medium (ABM) and hemolymph from the day 1 of the fifth instar unparasitized *H. virescens* and P1 for ABM and hemolymph from the day 1 of the fifth instar parasitized *H. virescens* larvae. The media without the hemolymph was referred to as C (control). In the 'C' medium, the 50% hemolymph was replaced with ABM, which brought the total volume of ABM to 90%.

The difference between the liquid and semisolid rearing medium was that in the semisolid medium, the 40% of ABM was substituted with 20% of 2% agar and 20% of ABM. In the semisolid C medium, there was 70% ABM and 20% of 2% agar.

Incubation of parasitoid larvae in the rearing medium

The culture plate was kept at a 45°angle so that the diet remained at an angle in the lower part of the well facilitating the movement of the larvae out from the liquid diet. To each well, 100μl of the artificial rearing medium was extracted using a sterilized micropipette. For the new and early first instar larvae, 96 well culture plates (Nunclon)

and for the late first instar, early second and late second instar larvae, 48 well culture plates (Nunclon) were used. One larva per well was placed for all treatments and there were 10 replications for each treatment. The culture plates were sealed tightly using parafilm and kept at 29 ± 1° C. Top surface of the lid was covered with aluminum foil.

All the above described procedures were conducted in a laminar flow hood and all experimental materials were autoclaved at 121°C with a pressure of 1kg/cm^2 for 20 min.

Observations were carried out daily for 10 days. The length and width of the larvae before and after incubation were recorded using a dissection microscope at the 1.5 magnification. The size was then converted to mm using the correction factor for 1.5 magnification (0.0689). The number of larvae molted from one instar to the next were also recorded. The instars of the larvae were determined according to the method of Lewis & Vinson (1968).

Osmotic pressure

The osmotic pressure of the rearing media before and after incubation was measured using a Vapor Pressure Osmometer (VAPRO Model 5520). The osmotic pressure was measured for 4 random samples from each type of rearing media.

Protein quantification

Protein concentration of hemolymph samples was determined by the method of Bradford (1976) using the Coomassie ® Plus Protein Assay Reagent Kit, (Cat. no. 23236, Pierce, 3747 N. Meridian Road, P.O. Box 117, Rockford, IL 61105) with bovine serum albumin as a standard.

2D gel electrophoresis

Based on the protein assay, the hemolymph volume to contain a minimum of 246 μg of protein was calculated which was then desalted and precipitated with ice cold acetone (Dunn, 1987). The precipitated sample was delivered to the Protein Chemistry Laboratory (Biochemistry and Biophysics Department, TAMU) to perform the 2D gel electrophoresis.

Statistical analysis

Statistical analyses were performed using JMP® Statistical Discovery Software, Version 4 (2001 SAS Institute Inc. Cary, NC, USA version 4). The data was normalized using square root transformation before analysis. A three way ANOVA (full factorial model) was used to evaluate the effect of the three factors: 1) the age of the larvae (new first instar, early first instar, late first instar, early second instar and late second instar), 2) type of rearing media i.e. C (ABM without hemolymph), UP1, UP3 and UP5 (ABM with hemolymph from unparasitized day 1, day 3, and day 5 fifth instar host larvae), and P1, P3 and P5 (ABM with hemolymph from parasitized day 1, day 3, and day 5 fifth instar host larvae); and, 3) the forms of the rearing media – the liquid and semisolid (media with and without agar) on the growth (length and width gain) of the new first instar, early first instar, late first instar, early second instar and late second instar *T. nigriceps* larvae. If the three factors were significant and the interaction terms between these factors were not significant, the means were separated on each factor by two way ANOVA and a multiple comparison analysis (Tukey Honestly Significant Difference mean separation technique). The null hypotheses were that 1) there was no effect of

these factors on the growth of the larvae separately and 2) the interactions among these three factors had no effect on the growth of the larvae. Since the interaction terms were significant in the above model, the effect of the two factors (type of media and form of media) was analyzed separately on the growth of the larvae within each particular age group.

A paired t-test was done to compare the osmotic pressure of the seven different types of rearing media before and after the incubation with the parasitoid larvae. One way ANOVA was used to analyze the protein concentration of the hemolymph of the day 1, 3 and 5 of the fifth instar parasitized and unparasitized *H. virescens* larvae.

Data collection from the 2D gels

The gels of the day 1, day 3, and, day 5 of the fifth day hemolypmh of the unparasitized (UP1, UP3 and UP5) and parasitized (P1, P3, and P5) fifth instar larvae of *H. virescens* were scanned. Then, each gel was divided into 10 equal parts vertically, and 7 parts horizontally to represent each molecular weight of the known standard proteins used in the gel. By thorough visual observation, the presence or absence of each spot in each row was observed and marked in the same location in a blank grid of the same size. Thus a comparison was made between 1) UP1, UP3 and UP5 (unparsitized); 2) P1, P3 and P5 (parasitized) and 3) between the hemolymph of the same aged larvae of unparasitized vs. parasitized fifth instar *H. virescens* larvae, i.e. UP1 vs. P1, UP3 vs. P3, and, UP5 vs. P5.

III RESULTS

Growth (increase in length and width)

Seven different types of rearing media (C, UP1, P1, UP3, P3, UP5 & P5) were tested in both liquid (without agar) and semisolid (with agar) form. Five different ages of the parasitoid larvae (new first instar, early first instar, late first instar, early second instar and late second instar) were incubated in each of these different rearing media. There were a total of 700 (350 for semisolid and 350 for liquid form for each type of rearing media) observations with 70 in each of the age group of the larvae. The statistical analysis of all the three factors (age of larvae, type of rearing media, form of media – liquid or semisolid) for the 700 observations together showed that all the three factors (age of larva - df = 4, F Ratio = 468.0892, P<0.0001, type of media – df = 6, F Ratio = 27.5924 and P<0.0001, form of media – df = 1, F Ratio = 39.1267, P < 0.0001), and their interactions (age of larva * type of media – df = 24, F Ratio = 3.6831, P < 0.0001, age of larva* form of media – df = 4, F Ratio = 23.9953, P <0.0001, type of media * form of media – df = 6, F Ratio = 3. 3035, P < 0.0033, age of larva* type of media* form of media – df = 24, F Ratio = 3.7371 and P <0.001) had a significant effect in length gain (Table 1).

The width increase was also significantly affected by these three factors (age of larva – df = 4, F Ratio = 529.7771, P < 0.0001, type of media – df = 6, F Ratio = 34.4731, P < 0.0001, form of media – df = 1, F Ratio = 2.1036, P <0.0001) and the interactions between these three factors (age of larva * type of media – df = 24,

F Ratio= 3.8645, P < 0.0001, age of larva* form of media – df = 4, F Ratio = 13.0600, P <0.0001, type of media * form of media – df = 6, F Ratio = 7.0440, P < 0.0001, age of larva* type of media* form of media – df = 24, F Ratio = 3.8856 and P <0.001) (Table 2).

Table 1.Three way ANOVA describing the main effects of age of larva, type of rearing media and form of rearing media on the growth (increase in length) of *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	69	220.38176	3.19394	34.2866	<0.0001
Error	630	58.68710	0.09315		
Corrected total	699	279.06886			

Source	DF	Sum of Squares	F Ratio	Prob>F
Age of larva	4	174.41776	468.0892	<.0001
Type of rearing media	6	15.42205	27.5924	<.0001
Form of rearing media	1	3.64481	39.1267	<.0001
Age of larva*type of rearing media	24	8.23432	3.6831	<.0001
Age of larva*form of rearing media	4	8.60569	23.0953	<.0001
Type of rearing media*form of rearing media	6	1.84638	3.3035	0.0033
Age of larva*type of rearing media*form of rearing media	24	8.35506	3.7371	<.0001

Table 2. Three way ANOVA describing the main effects of age of larva, type of rearing media, form of rearing media on the growth (increase in width) of *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	69	99.55412	1.44281	38.7628	<0.0001
Error	630	23.44962	0.03722		
Corrected total	699	123.00373			

Source	DF	Sum of Squares	F Ratio	Prob>F
Age of larva	4	78.876634	529.7771	<.0001
Type of rearing media	6	7.698864	34.4731	<.0001
Form of rearing media	1	2.103613	56.5159	<.0001
Age of larva*type of rearing media	24	3.452244	3.8645	<.0001
Age of larva*form of rearing media	4	1.944453	13.0600	<.0001
Type of rearing media*form of rearing media	6	1.573144	7.0440	<.0001
Age of larva*type of rearing media*form of rearing media	24	3.885600	4.3496	<.0001

Growth of the new first instar larvae

Increase in length

The new first instar (2d) larvae showed minimum growth in all the media. The average length of the 140 larvae incubated in the semisolid (70 larvae) and liquid media (70 larvae) before incubation ranged between 0.73-0.76 mm, and 10 days after incubation ranged between 0.73-86 mm (Table 3). The highest % of length increase was

Table 3. Average length increase (in mm) of the new first instar, early first instar, late first instar, early second instar, and late second instar *T. nigriceps* larvae before and after incubation in the semisolid and liquid form of the seven types of artificial rearing media

Length		In semisolid (in mm)			In Liquid (in mm)	
	Before	after	Increase %	Before	after	increase %
New first instar larvae						
UP1	0.7579	0.7786	3.64	0.7579	0.7855	3.64
P1	0.7372	0.7786	5.60	0.7454	0.7980	7.06
UP3	0.7303	0.7924	8.46	0.7303	0.7992	9.43
P3	0.7303	0.8199	12.26	0.7372	0.8268	12.15
UP5	0.7510	0.8061	7.34	0.7303	0.7717	5.66
P5	0.7510	0.8613	14.67	0.7303	0.8199	12.27
C	0.7648	0.7992	4.51	0.7097	0.7303	2.91
Early first instar larvae						
UP1	1.1024	1.1989	8.75	1.0889	1.1644	6.96
P1	1.1024	1.2402	12.50	1.0612	1.1713	10.38
UP3	1.0886	1.2953	18.99	1.1162	1.2678	13.58
P3	1.0817	1.3918	28.66	1.1093	1.3780	24.22
UP5	1.1300	1.3780	21.95	1.1162	1.2540	12.35
P5	1.1093	1.4193	27.95	1.1231	1.3573	23.31
C	1.1024	1.2471	13.13	1.1024	1.2264	11.25
Late first Instar larvae						
UP1	1.3436	1.8190	35.38	1.3091	1.5640	19.47
P1	1.3150	2.3908	81.66	1.3160	1.7570	33.51
UP3	1.3091	2.7422	109.47	1.3367	2.1635	61.99
P3	1.3091	4.2511	224.73	1.3573	2.3633	74.11
UP5	1.3160	2.4528	86.37	1.3298	2.1704	63.21
P5	1.3160	6.3526	382.72	1.3367	2.2737	70.10
C	1.2953	2.3357	80.32	1.3160	1.8121	37.70
Early second instar						
UP1	3.5484	5.2364	45.57	3.5621	5.5396	55.51
P1	3.5484	5.2709	48.54	3.5621	6.3870	79.30
UP3	3.5484	6.5111	83.49	3.5621	6.7591	89.75
P3	3.5552	6.9934	96.71	3.5552	6.9176	94.57
UP5	3.5484	6.3388	78.64	3.5484	5.8358	64.47
P5	3.5552	7.2001	102.52	3.5415	6.9038	94.94
C	3.5552	6.4559	81.59	3.5484	6.3595	79.22
Late second instar						
UP1	7.1932	8.6194	19.82	7.1656	7.3723	2.88
P1	7.2069	8.4885	17.78	7.1725	7.3792	2.88
UP3	7.1863	7.8891	9.78	7.1518	7.9097	10.60
P3	7.2069	9.0052	24.95	7.1656	8.3714	16.83
UP5	7.1932	8.6194	19.83	7.1863	8.5092	18.41
P5	7.1863	8.9501	24.54	7.1656	8.3025	15.87
C	7.1932	8.6883	20.78	7.1794	7.7375	7.77

observed in P5 semisolid media (14.67%) and the lowest % was in liquid C media (2.91%) (Table 3).

The length gain of the new first instar larvae was significantly affected only by the type of the rearing media in which the larvae were incubated (P=0.0014) while the other factor – form of media, did not have a significant effect (P= 0.8189). The interactions between these two factors (type of rearing media and form of rearing media) also had a significant effect on the length gain of the larvae (P= 0.0428) (Table 4). In other words, the larval length gain was affected only by the type of rearing media it was in regardless of whether the rearing media was in liquid or semisolid form.

Table 4. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in length) of the new first instar (2d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	0.7291190	0.056086	2.8266	0.0014
Error	126	2.5000932	0.019842		
Corrected Total	139	3.2292122			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	0.46048977	3.8680	0.0014
Form of rearing media	1	0.00104468	0.0526	0 .8189
Type of rearing media*form of rearing media	6	0.26758453	2.2476	0.0428

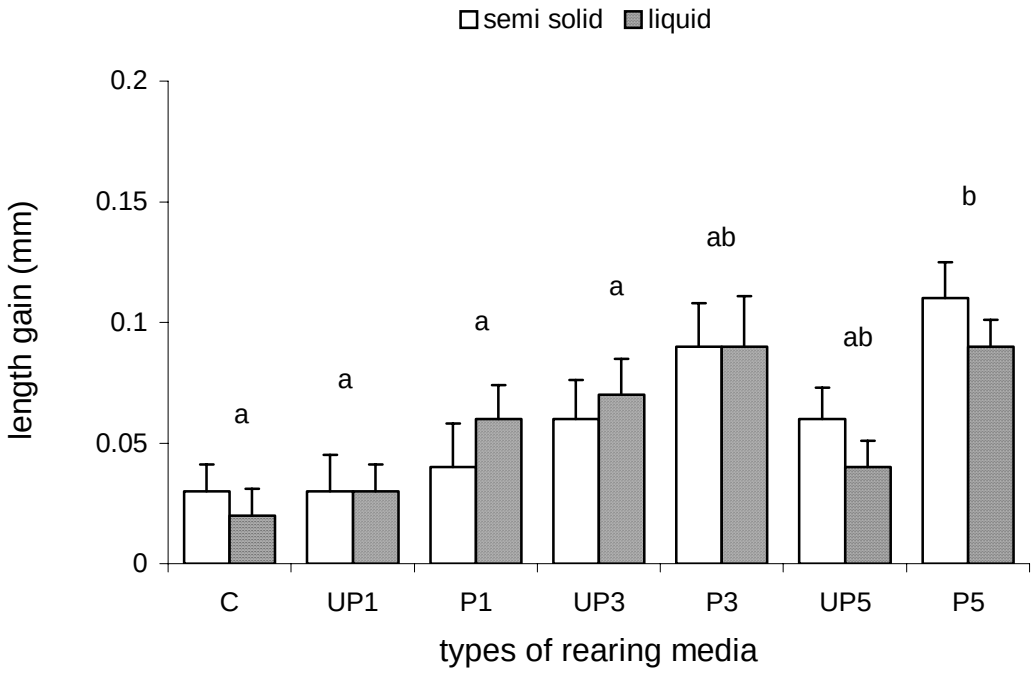

Fig. 1a. Increase in length of *T. nigriceps* new first instar (2d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

The length increase in C (ABM without any hemolymph) was significantly different only from P5. Between the unparasitized media –UP1, UP3 and UP5, the length gain was not significantly different from each other. Between the parasitized media – P1, P3 and P5; there was significant difference between P1 and P5, but not between P3 and P5. Comparing between the rearing media with unparasitized and parasitized hemolymph of the same age (UP1 Vs P1, UP3 Vs P3 and UP5 Vs P5), there was no significant difference in length increase between any of the pair (Fig. 1a).

Increase in width

The average initial width of the total number of larvae (140) was between 0.23-0.27 mm and after the 10 days observation period was between 0.25-0.32 mm (Table 5). The highest % of width increase in the P5 (27.78%) and the lowest % was in UP1 liquid media (0%) (Table 5).

The increase in width was significantly affected only by the type of rearing media (P= 0.0003) in which the parasitoid larva was incubated, but not by the form of rearing media (P=0.0648), or the interaction between the two factors (P=0.4276) (Table 6).

The width gain in C was significantly different from UP3, P3, and P5. Between the unparasitized media, UP1 was significantly different from UP3, but not different from UP5. Among the parasitized media, there were no significant differences in width gain between the three media (P1, P3 and P5). In comparing the unparasitized and parasitized media with the same age of hemolymph, there were no significant

Table 5. Average width increase (in mm) of the new first instar, early first instar, late first instar, early second instar, and late second instar *T. nigriceps* larvae before and after incubation in the semisolid and liquid form of the seven types of artificial rearing media

Width	In semisolid (in mm)			In Liquid (in mm)		
	Before	after	Increase %	Before	after	increase %
New first instar larvae						
UP1	0.2618	0.2687	2.63	0.2687	0.2687	0
P1	0.2480	0.2687	8.35	0.2499	0.2750	10.04
UP3	0.2343	0.2756	17.63	0.2343	0.2756	17.63
P3	0.2343	0.2894	23.52	0.2480	0.2963	19.44
UP5	0.2480	0.2894	16.65	0.2480	0.2687	8.35
P5	0.2480	0.3169	27.78	0.2618	0.2875	9.82
C	0.2549	0.2756	10.24	0.2274	0.2480	9.10
Early first instar larvae						
UP1	0.3445	0.3790	10.01	0.3445	0.3790	10.00
P1	0.3445	0.3790	10.01	0.3445	0.3721	8.01
UP3	0.3445	0.4065	18.00	0.3514	0.3996	13.72
P3	0.3445	0.4065	18.00	0.3445	0.4065	18.00
UP5	0.3445	0.4000	15.99	0.3445	0.3858	11.99
P5	0.3445	0.4065	18.00	0.3445	0.4065	20.00
C	0.3445	0.3858	11.99	0.3445	0.3858	11.99
Late first instar larvae						
UP1	0.4134	0.5236	26.66	0.4134	0.4479	8.35
P1	0.4134	0.6408	53.88	0.4134	0.4823	16.67
UP3	0.4134	0.7441	81.52	0.4134	0.5994	45.00
P3	0.4134	1.3642	231.74	0.4134	0.6132	48.33
UP5	0.4134	0.6683	61.66	0.4134	0.6063	46.67
P5	0.4134	1.9223	365.00	0.4134	0.5925	43.32
C	0.4134	0.6477	56.68	0.4134	0.5099	23.34
Early second instar						
UP1	0.8613	1.4601	69.59	0.8750	1.4538	66.15
P1	0.8613	1.5434	79.19	0.8750	1.9085	118.11
UP3	0.8613	1.9430	125.59	0.8750	2.0463	133.86
P3	0.8681	2.1979	153.19	0.8681	2.1704	150.00
UP5	0.8613	1.8465	114.40	0.8613	1.6743	94.39
P5	0.8681	2.1979	153.19	0.8544	2.1497	151.60
C	0.8681	1.7078	118.26	0.8613	1.8879	119.19
Late second instar						
UP1	2.3702	2.6458	3.83	2.3426	2.4322	3.82
P1	2.4184	2.6458	3.09	2.3495	2.4391	3.81
UP3	2.3495	2.5355	7.92	2.3288	2.6527	13.90
P3	2.3839	2.6389	10.69	2.3426	2.8180	20.29
UP5	2.3702	2.6044	9.89	2.3633	2.8731	21.58
P5	2.3633	2.6251	11.08	2.3426	2.8869	23.23
C	2.3702	2.4942	5.23	2.3426	2.4942	6.47

Table 6. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in width) of the new first instar (2d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	0.5813650	0.044720	2.8620	0.0012
Error	126	1.9688192	0.015626		
Corrected Total	139	2.5501842			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	0.43326703	4.6214	0.0003
Form of rearing media	1	0.05422792	3.4705	0.0648
Type of rearing media*form of rearing media	6	0.093870006	1.0012	0.4276

differences between any of the two i.e. between UP1 and P1, UP3 and P3 and UP5 and P5 (Fig. 1b).

Growth of early first instar larvae

Increase in length

The early first instar (4d) larvae grew much better in all the media than the new first instar larvae (2d). The average length of the early first instar larvae before and after incubation ranged between 1.06-1.13 mm and 1.16-1.42 mm, respectively (Table 3). The highest % of length increase was observed in semisolid P3 medium (28.66%) and the lowest was in UP1 liquid medium (6.96%) (Table 3).

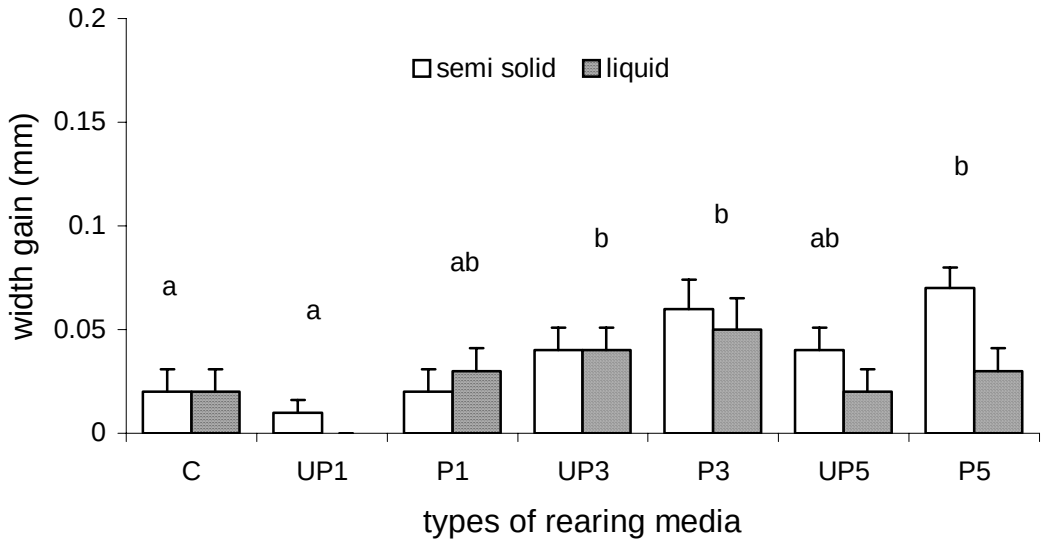

Fig. 1b. Increase in width of *T. nigriceps* new first instar (2d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Table 7. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in length) of the early first instar (4d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	1.4417992	0.110908	3.3262	0.0002
Error	126	4.2012991	0.033344		
Corrected Total	139	5.6430982			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	0.76829088	3.8403	0.0015
Form of rearing media	1	0.32694969	9.8055	0.0022
Type of rearing media*form of rearing media	6	0.34655861	1.7323	0.1187

Both factors, type of rearing media and form of rearing media, had a significant effect on the length gain of the larvae (P= 0.0015, P= 0.0022 respectively). However, the interaction terms between these two factors did not have a significant effect on the length gain of the early first instar larvae (P= 0.1187) (Table 7).

The length gain in C medium was not significantly different from any other media. Among the three media with unparasitized hemolymph (UP1, UP3 and UP5), there were no significant differences in length increase. Among the three media with the parasitized hemolymph (P1, P3 and P5), there was significant difference in length gain between P1 and P5, but not between P1 and P3 or between P3 and P5. Comparing the media with the same age of unparasitized and parasitized hemolymph added, there

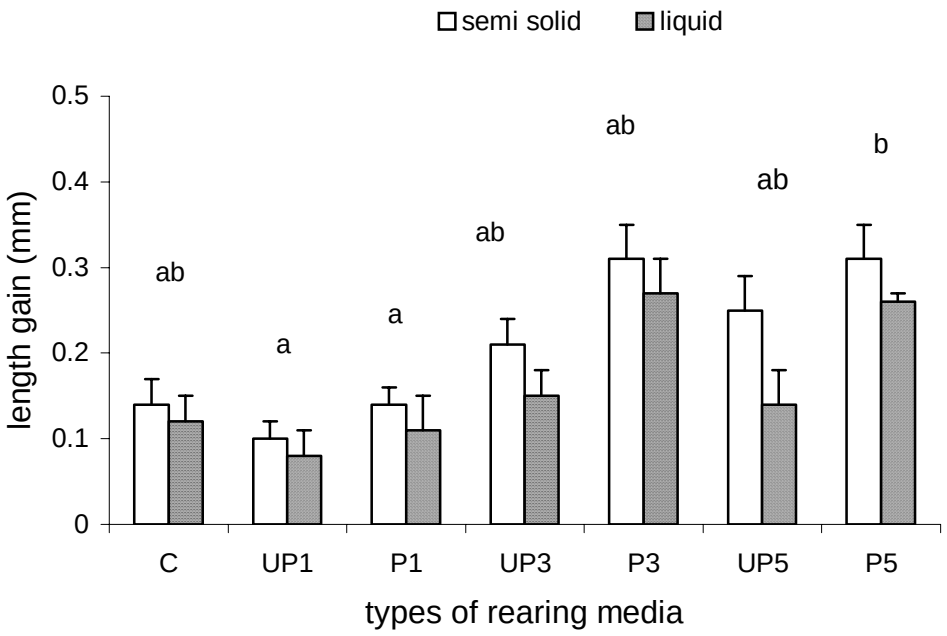

Fig. 2a. Increase in length of *T. nigriceps* early first instar (4d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

were no significant differences among any of the three sets (i.e. between UP1 and P1, UP3 and P3, and between UP5 and P5). Though the form of media had a significant effect on the length increase in the whole model, there was no significant difference between the semisolid and liquid form within any of the seven type of rearing media, e.g. between the semisolid Vs. liquid form of C or the semisolid Vs liquid form of UP1 etc. (Fig. 2a).

Increase in width

The average initial width of the early first instar larvae before incubation was 0.34 mm and after the observation period, the final width ranged between 0.38-0.41 mm among the total of 140 larvae incubated altogether in the semisolid and liquid media (Table 5). The highest % was in P5 liquid media (20%) and the lowest was in liquid P1 media (8.01%) (Table 5).

Only the type of rearing media had a significant effect on width gain (P=0.0092) of the early first instar larva. Form of media did not affect the width gain significantly (P=0.1269). The interaction between these two factors was also not significant enough to have an effect on width gain (P=0.9734) (Table 8).

The width increase in larvae incubated in C was not significantly different from any other media. Among the unparasitized group of rearing media (UP1, UP3, and UP5), there were no significant differences in width increase. Among the parasitized group, the width gain in P1 was significantly different from P3 and P5, but no difference between P3 and P5 was found. Between the unparastized and parasitized media, there

Table 8. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in width) of the early first instar (4d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	0.2920000	0.022462	1.6570	0.0784
Error	126	1.7080000	0.013556		
Corrected Total	139	2.0000000			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	0.24300000	2.9877	0.0092
Form of rearing media	1	0.03200000	2.3607	0.1269
Type of rearing media*form of rearing media	6	0.01700000	0.0290	0.9734

were no significant differences in width gain between UP1and P1, UP3 and P3, and between UP5 and P5 (Fig. 2b).

Growth of late first instar larvae

Increase in length

Late first instar (6d) larvae grew better in the semisolid media of all the seven types of rearing media than their counterparts in the liquid media. The average initial length of the larvae before incubation ranged between 1.30-1.36 mm and the final average length after the observation period ranged between 1.56-6.32 mm (Table 3). The highest % of length increase was in semisolid P5 (382.72%) and the lowest % was in UP1 liquid media (19.47%) (Table 3).

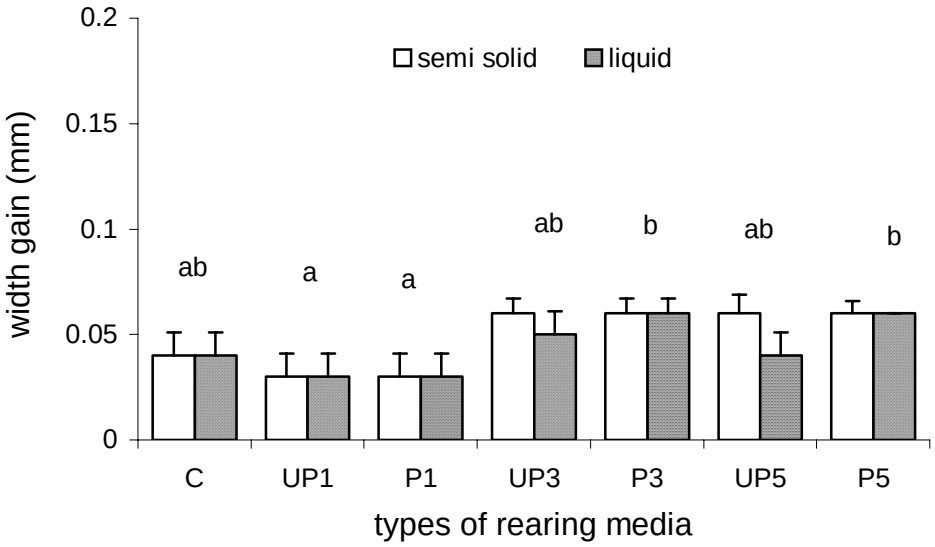

Fig. 2b. Increase in width of *T. nigriceps* early first instar (4d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Table 9. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in length) of the late first instar (6d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	26.710918	2.05469	15.3502	<0.0001
Error	126	16.865576	0.13385		
Corrected Total	139	43.576494			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	13.051244	16.2506	<0.0001
Form of rearing media	1	8.473660	63.3053	<0.0001
Type of rearing media*form of rearing media	6	5.186014	6.4573	<0.0001

For the late first instar larvae, both factors (type of rearing media and form of rearing media) and the interaction between the two factors had a significant effect on the length gain of the larvae (Table 9). The length gain in C was significantly different from that in UP3, P3, UP5, and P5. Among the three media with the unparasitized hemolymph, the length gain in UP1 was significantly different from UP3 and UP5, but there were no significant differences between UP3 and UP5. Between the parasitized group of media, P3 and P5 were significantly different from P1; however, there was no significant difference between P3 and P5. Comparing the length increase in media with the unparasitized and parasitized hemolymph of the same age, there was a significant

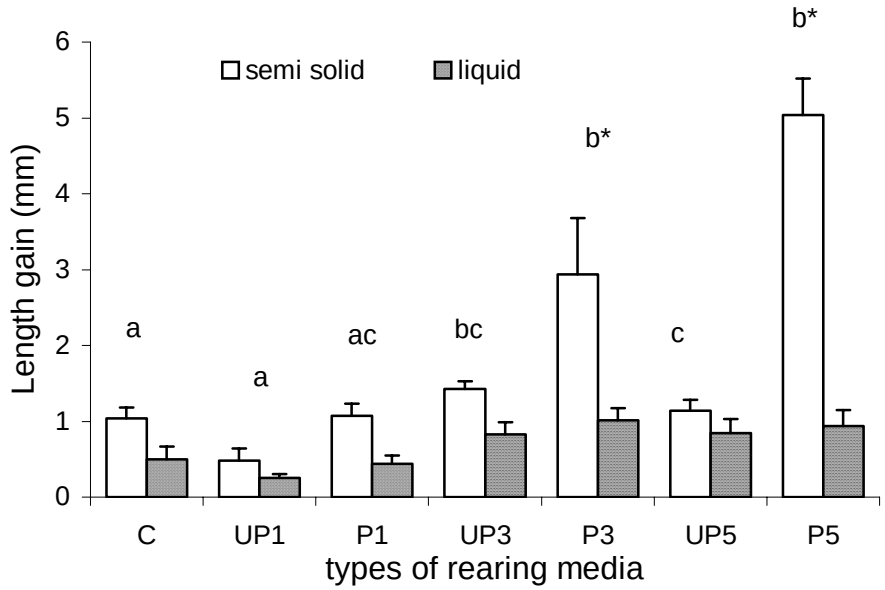

Fig. 3a. Increase in length of *T. nigriceps* late first instar (6d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

* denotes a significant difference ($P<0.05$) between the semisolid and liquid form of media within a particular type of media. C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

difference only between UP5 and P5. The larval length increase was significantly different between the semisolid and liquid form of media in P3 and P5 (Fig. 3a).

Increase in width

The average width of the late first instar larvae before incubation was 0.41mm and after the incubation period ranged between 0.45-1.92 mm (Table 5). The width increase was the maximum in the semisolid P5 media (365%) and the minimum was in the liquid UP1 media (66.15%) (Table 5).

Like the length increase, width gain of the late first instar larvae was significantly affected by both factors (type of media and form of media) and the interactions between these two factors ($P<0.0001$). In other words, the width increase of the larva was dependent on what type of media it was in, whether the media was in liquid form or in semisolid form; and also whether these two factors were present at the same time (Table 10).

The width increase in C was significantly different from P3 and P5, but not different from UP1, P1, UP3 and UP5. Between the media with unparasitized hemolymph, UP1 was significantly different from UP3 and UP5, but no significant difference between UP3 and UP5. Likewise, between the media with parasitized hemolymph, the larval width gain in P1 was significantly different from that in P3 and P5, but there was no significant difference between P3 and P5 (Fig. 3b). Comparing the two groups of media with the same aged unparasitized and parasitized host hemolymph, the significant difference was only between UP5 and P5, as in length increase. There

Table 10. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in width) of the late first instar (6d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	11.281015	0.867770	14.7644	<0.0001
Error	126	7.405575	0.058774		
Corrected Total	139	18.686589			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	5.7827389	16.3981	<0.0001
Form of rearing media	1	3.3477032	56.9585	<0.0001
Type of rearing media*form of rearing media	6	2.1505725	6.0984	<0.0001

were significant differences in width gain between the semisolid and liquid form of UP3, P3 and P5 media (Fig. 3b).

Growth of early second instar larvae

Increase in length

For the early second instar (8d) larvae, the average initial length of the larvae before incubation ranged between 3.54-3.56 mm and the average final length after the observation period ranged between 5.24-7.20 mm (Table 3). The highest % of length increase was in semisolid P5 (102.52%) and the lowest was in semisolid UP1 media (45.57%) (Table 3).

The length increase was significantly affected only by the type of the rearing media (P=0.0002) and not by the form of the rearing media i.e. whether the media was in liquid or semisolid form (P=0.9230). Also, the interaction between these two factors

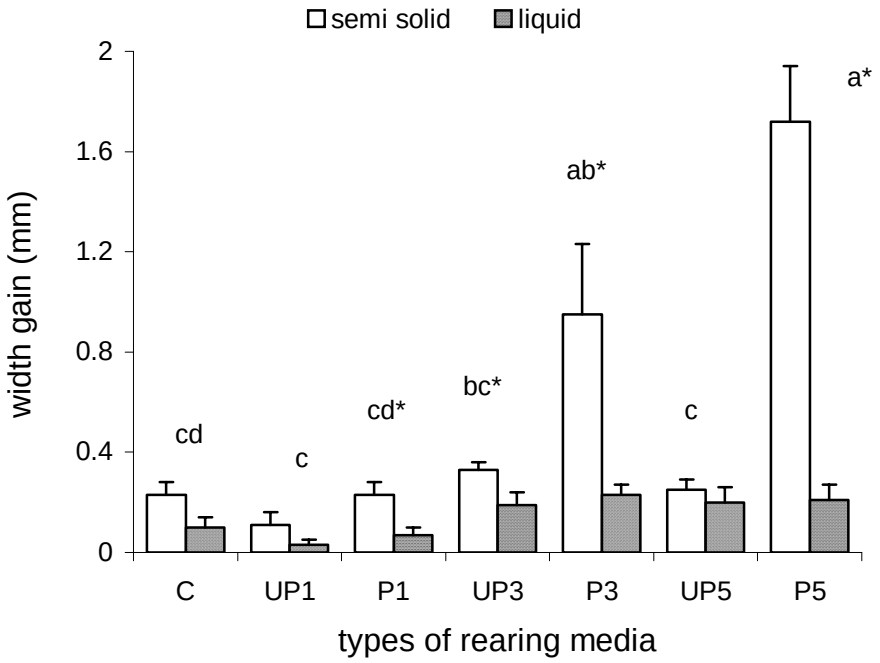

Fig. 3b. Increase in width of *T. nigriceps* late first instar (6d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

* denotes a significant difference ($P<0.05$) between the semisolid and liquid form of media within a particular type of media. C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Table 11. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in length) of the early second instar (8d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	5.845976	0.449690	2.5379	0.0039
Error	126	22.325537	0.177187		
Corrected Total	139	28.171513			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	5.0300553	4.7314	0.0002
Form of rearing media	1	0.0016617	0.0094	0.9230
Type of rearing media*form of rearing media	6	0.8142593	0.7659	0.5981

(type of larvae and the form of media) had no significant effect on the length gain (P=0.5981) (Table 11).

The larvae grew well in all media. The larval length gain in C was not significantly different any of the other media. Among the UP media, UP3 was significantly different from UP1 and UP5, but no significant difference between UP1 and UP5. Between the P media, the length increase in P1 was significantly different from the length increase in P3 and P5; however no significant difference was shown between P3 and P5. Comparing the UP media with the P media with the same aged hemolymph, the significant difference was only between UP5 and P5 (Fig. 4a).

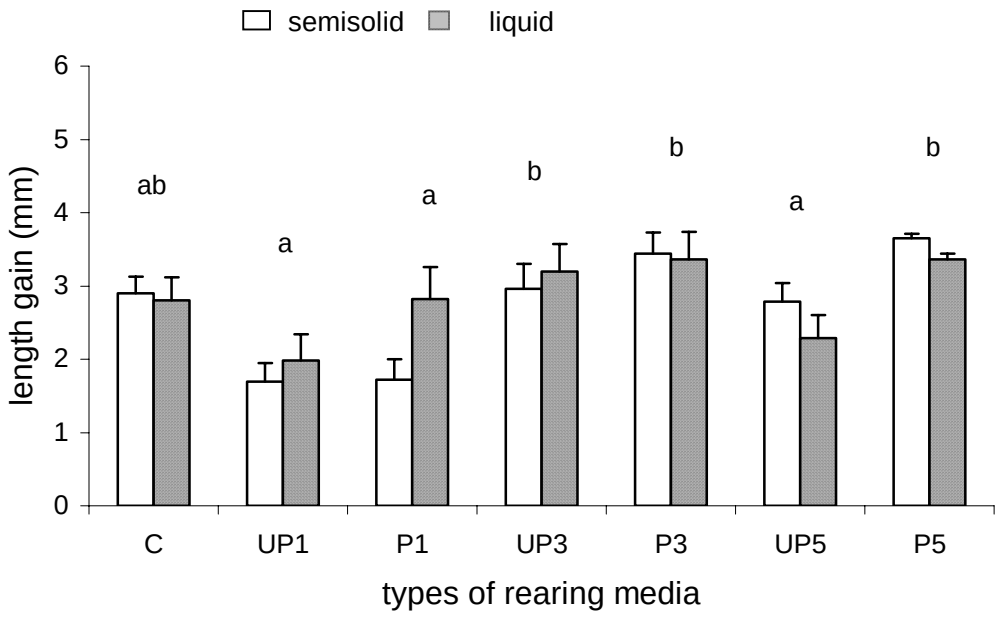

Fig. 4a. Increase in length of *T. nigriceps* early second instar (8d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Increase in width

The initial and final increase in width of the early second instar larvae before and after incubation in the artificial rearing media ranged between 0.86-0.88mm and 1.45-2.18 mm, respectively (Table 5). Likewise, the highest and lowest % of width increase were in semisolid P5 media (153.19%) and in liquid UP1 media (66.15%), respectively (Table 5).

Similar to length increase, in the width gain, only the type media ($P<0.001$) and not the form of media ($P=0.2737$) or the interaction between the two factors ($P=0.6909$) had a significant effect for the early second instar larvae (Table 12).

The width gain in C media was not significantly different from any of the other media as in length gain. In the media with the unparasitized hemolymph, the width gain in UP1 and UP5 were significantly different from UP3. In the media with the parasitized hemolymph, the width gain in P1 was significantly different from P3 and P5. However, there was no significant difference between P3 and P5. Comparing the unparasitized and parasitized media with the same aged hemolymph, the significant differences in width gain were only between UP5 and P5 (Fig. 4b).

Growth of late second instar larvae

Increase in length

The average initial length of the late second instar (10d) larvae before incubation ranged between 7.15-7.21 mm and the final average length after the observation period ranged between 7.37-8.95 mm (Table 3). The highest and lowest % of length increase

Table 12. Two way ANOVA on the main effects of type of rearing media and form of rearing media on the growth (increase in width) of the early second instar (8d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	2.898567	0.222967	3.8710	<0.0001
Error	126	7.257485	0.057599		
Corrected Total	139	10.156052			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	2.6046922	7.5368	<0.0001
Form of rearing media	1	0.0696280	1.2088	0.2737
Type of rearing media*form of rearing media	6	0.224271	0.6489	0.6909

were recorded in the semisolid P3 (24.95%) and in liquid UP1 and P1media (2.88%) respectively.

The length increase of the late second instar larvae were significantly affected by both factors – type of media and form of media, and also by the interactions between these two factors (P<0.0001) (Table 13).

The length increase in C was not significantly different from any other media. Between the three unparasitized media, UP1 and UP3 were significantly different from UP5. Among the three parasitized media, the length gain in P1 was significantly different from that in P3 and P5. However, there were no significant differences between P3 and P5. Comparing the length gain between UP and the P media with the same aged host hemolymph, the significant difference was only between UP3 and P3. Between the

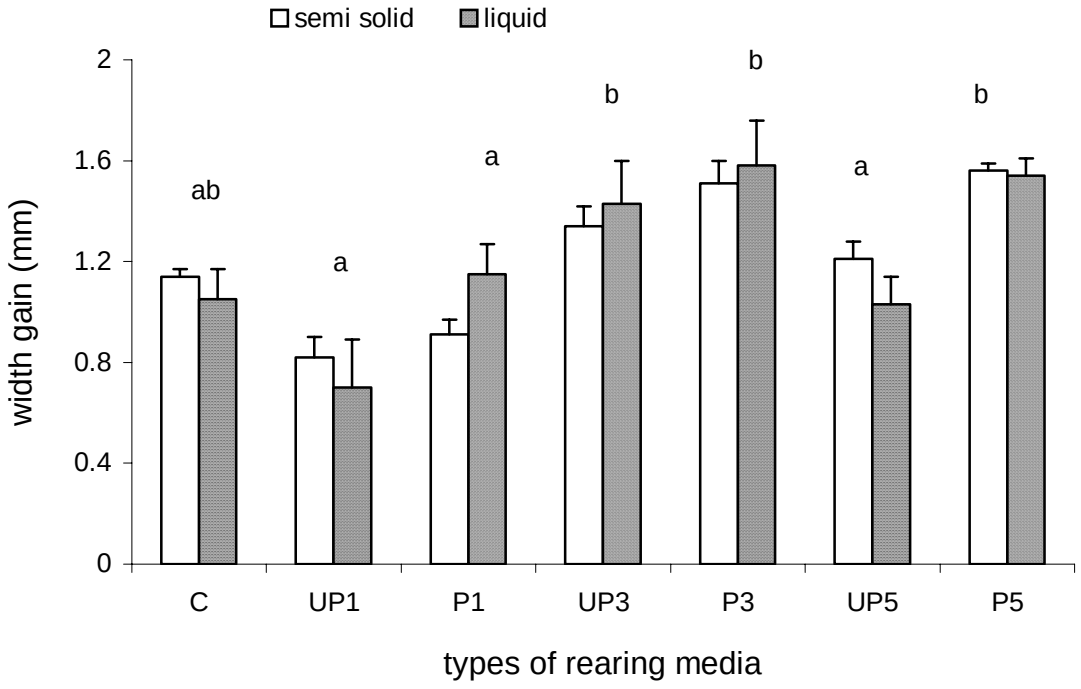

Fig. 4b. Increase in width of *T. nigriceps* early second instar (8d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Table 13. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in length) of the late second instar (10d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	11.361632	0.873972	8.6068	<0.0001
Error	126	12.794593	0.101544		
Corrected Total	139	24.156225			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	4.3465779	7.1341	<0.0001
Form of rearing media	1	3.4447159	33.9233	<0.0001
Type of rearing media*form of rearing media	6	3.5863346	5.8863	<0.0001

semisolid and liquid form, the significant difference in length gain was in C, UP1, and P1 media (Fig. 5a).

Increase in width

The average initial width of the late second instar larvae before incubation ranged between 2.34-2.42 mm and the final average width after the observation period ranged between 2.43-2.89 mm (Table 5). The highest and lowest % of width increase were recorded in the liquid P5 (23.23%) and in semisolid P1 media (3.09%) respectively (Table 5).

Like in length increase, width increase was also significantly affected by both factors – type of rearing media and form of rearing media and also by the interactions between these two factors ($P<0.001$, $P=0.004$ and $P<0.001$ respectively) (Table 14).

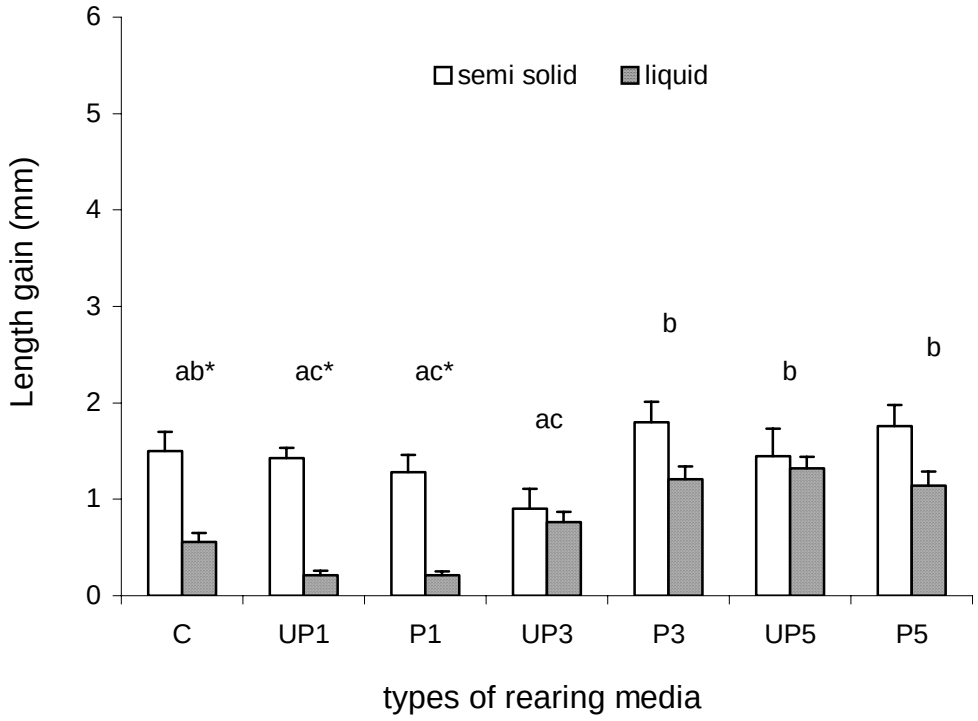

Fig. 5a. Increase in length of *T. nigriceps* late second instar (10d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

* denotes a significant difference ($P<0.05$) between the semisolid and liquid form of media within a particular type of media. C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Table 14. Two way ANOVA describing the main effects of type of rearing media and form of rearing media on the growth (increase in width) of the late second instar (10d) *T. nigriceps* larvae in vitro

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Model	13	5.635940	0.433534	10.6904	<0.0001
Error	126	5.109737	0.040553		
Corrected Total	139	10.745677			

Source	DF	Sum of Squares	F Ratio	Prob>F
Type of rearing media	6	2.0853024	8.5702	<0.0001
Form of rearing media	1	0.5443402	13.4228	<0.0004
Type of rearing media*form of rearing media	6	2.9730372	12.2186	<0.0001

The width gain in C was significantly different from that in P3, UP5, and P5. In the unparasitized media, UP1 was significantly different from UP5, but there were no significant differences between UP1 and UP3 or between UP3 and UP5. In the parasitized media, the width increase in P1 was significantly different from P3 and P5; however, there was no significant difference between P3 and P5. Between the unparasitized and parasitized media with same aged host hemolymph, there were no significant differences between any of the group. Between the semisolid and liquid form, there were significant differences in width gain in C, P3, UP5, and P5 (Fig. 5b).

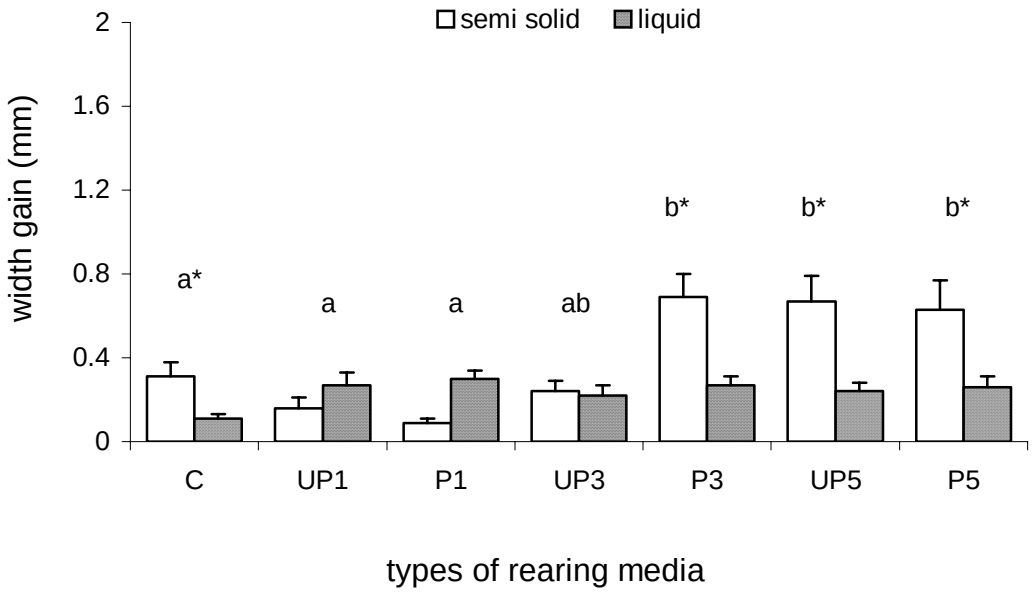

Fig. 5b. Increase in width of *T. nigriceps* late second instar (10d) larvae in vitro.

Means with a different letter are significantly different ($P<0.05$) between the different types of media.

* denotes a significant difference ($P<0.05$) between the semisolid and liquid form of media within a particular type of media. C = artificial rearing media without hemolymph, UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae.

Molting

New first instar (2d) larvae

No molting occurred in any type of media

Early first instar (4d) larvae

None of the larvae molted to second instar.

Late first instar (6d) larvae

Molting of the late first instar larvae occurred in all the media and in some cases a second molt occurred (molt to third instar). In the semisolid form of the rearing media, the highest percent of molting occurred in P5 (100%) and the lowest percent molting occurred in UP1, P1 and C (20%) (Fig. 6a). In the liquid form of media, both P3 and P5 media provided the highest % of molt (50%) while none of the larvae molted in UP1 and C media (Fig. 6b). The semisolid form of the diet produced a higher % of molting of the larvae than the liquid form for all the types of media. Even in semisolid form of C media, 20% of the larvae molted to a second instar. Further, 40% and 60% of the second instar molted to third instar, but only in P3 and P5 semisolid media, respectively. In addition, some behavioral changes, such as the larvae rolling over, and often shrinking, and expanding the body, were observed with the third instar larvae. These larval movements could be the preparation for pupation. However, no pupation occurred.

Early second instar (8d) larvae

Molting to a third instar occurred in all types of the semisolid media except in UP1 and P1- the highest percent of larvae that molted to third instar was in the P5 semi-

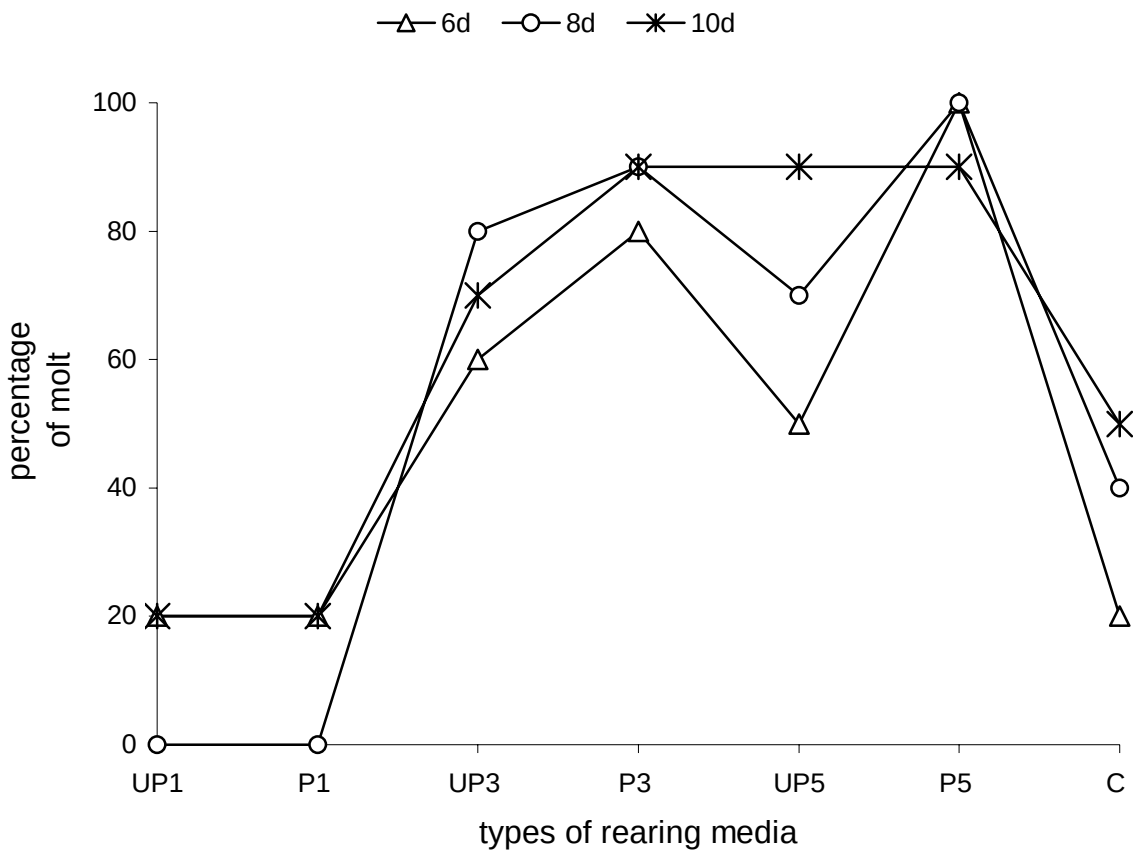

Fig. 6a. First molt (%) of *T. nigriceps* larvae in semisolid rearing media.

UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae, C = artificial rearing media without hemolymph.

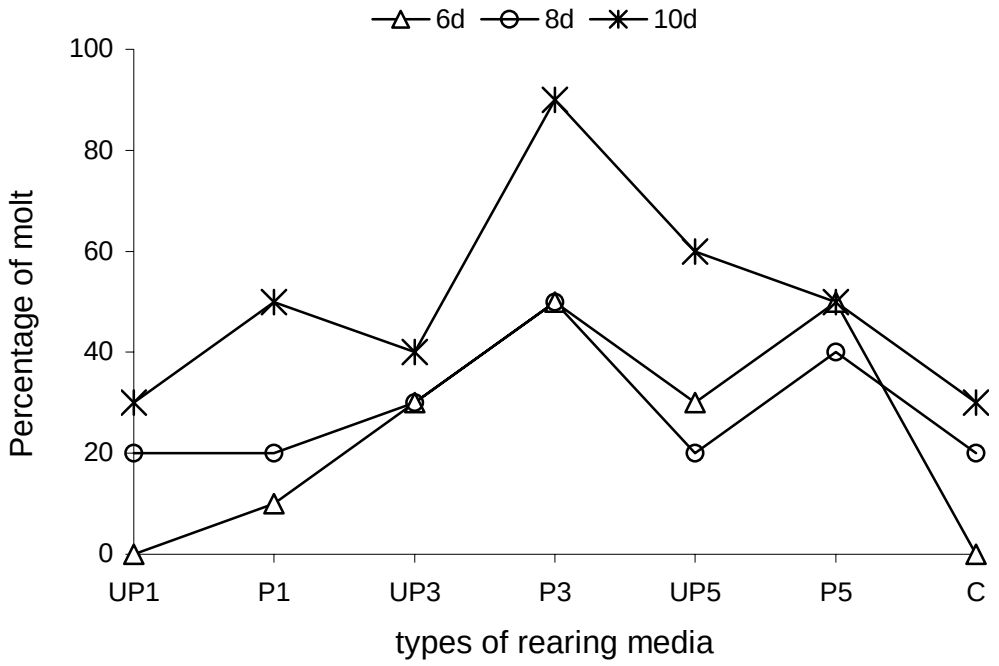

Fig. 6b. First molt (%) of *T. nigriceps* larvae in liquid rearing media.

UP1 = artificial rearing media with the hemolymph of the day 1 fifth instar unparasitized *H. virescens* larvae, P1= artificial rearing media with the hemolymph of the day 1 fifth instar parasitized *H. virescens* larvae, UP3 = artificial rearing media with the hemolymph of the day 3 fifth instar unparasitized *H. virescens* larvae, P3 = artificial rearing media with the hemolymph of the day 3 fifth instar parasitized *H. virescens* larvae, UP5 = artificial rearing media with the hemolymph of the day 5 fifth instar unparasitized *H. virescens* larvae and P5 = artificial rearing media with the hemolymph of the day 5 fifth instar parasitized *H. virescens* larvae, C = artificial rearing media without hemolymph.

solid medium (100%) and the lowest was in C medium (40%) (Fig. 6a). Although, molting occurred in all types of the liquid form of media; the percentage of larvae molted was lower when compared to semisolid form of the media (Fig. 6b). The highest % of second instar larvae molted in the liquid media was in P3 (50%) and the lowest % was in UP1, P1 and C (20%). In fact, the percentage of larvae molted to a third instar was more than double in the semisolid media compared to the liquid media. In the semisolid P5 media, 20% of the larvae that reached the third instar voided the gut producing meconium, which is a sign that the larvae were preparing for pupation. As described above concerning the development of late first instars, the resulting third instar larvae expressed the behavioral changes that could be interpreted as the preparation for pupation. In addition, a continuous exudate was observed oozing from the anterior oral area of some of these active third instar larvae. This may have been an attempt as the secretion of silk for cocoon formation, although neither a cocoon nor pupation was observed.

Late second instar (10d) larvae

Late second instar larvae incubated in either semisolid or liquid form of media, molted to the third instars in all the media. The highest % of molting were in semisolid P3, UP5 and P3 and in liquid P3 (90%) media and the lowest % were in semi solid UP1 and P1 (20%) (Fig 6a & b)). As observed with the third instar larvae above, behavioral changes such as constant rolling, shrinking, expansion of the body, and buccal secretion were observed. But no pupation was observed.

Table 15. Mean difference and std. error between the osmotic pressure (mOsm/kg) of the seven types of artificial rearing media before and after the incubation with the first instar larvae of *T. nigriceps*

Type of media	Before Incubation	After Incubation	Mean Difference ± Std Error	t-ratio	DF
New first instar (2d) larvae					
UP1	944.50[a]	969.00[a]	24.5 ±19.19	1.2	3
P1	971.25[a]	988.75[a]	17.5 ± 8.06	2.17	3
UP3	973.75[a]	994.50[b]	20.75 ± 5.14	4.04	3
P3	1017.00[a]	988.75[b]	-28.25 ± 2.39	-11.80	3
UP5	962.25[a]	980.00[a]	17.75 ± 9.4	1.88	3
P5	986.25[a]	991.00[a]	4.75 ± 3.28	1.45	3
C	1036.00[a]	991.00[b]	-45.00 ± 6.03	-7.47	3
Early first instar (4d) larvae					
UP1	944.50[a]	885.75[b]	-58.75 ± 9.38	-6.27	3
P1	971.25[a]	770.00[b]	-201.25 ± 6.65	-30.26	3
UP3	973.75[a]	846.00[b]	127.75 ± 6.93	-18.43	3
P3	1017.00[a]	702.25[b]	-314.75 ± 3.90	-80.65	3
UP5	962.25[a]	990.75[b]	28.50 ± 5.45	5.23	3
P5	986.25[a]	759.50 [b]	-226.75 ±3.64	-62.34	3
C	1036.00[a]	963.50[b]	-72.50 ± 2.47	-29.39	3

Table 15. Continued

Type of media	Before Incubation	After Incubation	Mean Difference ± Std Error	t-ratio	DF
Late first instar (6d) larvae					
UP1	944.50[a]	890.00[b]	-54.50 ± 6.67	-8.18	3
P1	971.25[a]	862.50[b]	-108.75 ± 10.18	-10.18	3
UP3	973.75[a]	760.50[b]	-213.25 ± 3.61	-59.00	3
P3	1017.00[a]	722.50[b]	-294.5 ± 21.12	-13.94	3
UP5	962.25[a]	765.50[b]	-196.75 ± 20.45	-9.62	3
P5	986.25[a]	638.25 [b]	-348.00 ± 7.67	-45.37	3
C	1036.00[a]	918.50[b]	-117.50 ± 7.90	-14.87	3

Means within rows with different letters are significantly different ($P<0.05$).

Survival

None of the larvae survived the 10d observation period. Normally the third instar larvae emerge from the host between 12-14 days after parasitization. The highest survival was observed in the late first instar larvae incubated in P3 and P5 semi solid media. In these two media, 10% (in P3) and 67% (in P5) of the third instar larvae developed from the late first instar larvae were alive for 9 days without further growth.

Comparison of osmotic pressure

Statistically, in all rearing media, there was a significant decrease between the osmotic pressure before and after incubation of the parasitoid larvae except those

incubated with the new first instar (2d) larvae (P<0.05). Only in P3 and C media, there were significant differences (decrease) in the osmotic pressure with the new first instar larvae (Table 15 & 16).

Table 16. Mean difference and std. error between the osmotic pressure (mOsm/kg) of the seven types of artificial rearing media before and after the incubation with second instar larvae of *T. nigriceps*

Type of media	Before Incubation	After Incubation	Mean Difference ± Std Error	t-ratio	DF
Early second instar (8d) larvae					
UP1	944.50[a]	730.25[b]	-214.25 ± 9.81	- 21.84	3
P1	971.25[a]	732.00[b]	-239.25 ± 6.76	-35.38	3
UP3	973.75[a]	734.00[b]	-239.75 ± 4.96	-48.38	3
P3	1017.00[a]	729.25[b]	-276.47 ± 3.54	-81.19	3
UP5	962.25[a]	833.5[b]	- 128.75 ± 3.42	-37.59	3
P5	986.25[a]	753.25 [b]	-233.00 ± 3.83	-40.84	3
C	1036.00[a]	982.00[b]	-54 ± 4.78	-11.30	3
Late second instar (10d) larvae					
UP1	944.50[a]	876.75[b]	-67.75 ± 8.34	-8.123	3
P1	971.25[a]	891.50[b]	-79.75 ± 7.52	-10.60	3
UP3	973.75[a]	870.50[b]	-103.25 ± 12.54	- 8.23	3
P3	1017.00[a]	856.75[b]	-160.25 ± 5.94	-27.00	3
UP5	962.25[a]	882.75[b]	- 79.5 ± 9.46	-8.41	3
P5	986.25[a]	864.00 [b]	-122.25 ± 7.55	-16.18	3
C	1036.00[a]	989.50[b]	-46.50 ± 4.87	- 9.54	3

Means within rows with different letters are significantly different (P<0.05).

Table 17. Means and std. error of the total protein quantities of the day 1, 3 and 5 hemolymph samples of the unparasitized and parasitized fifth instar larvae of *H. virescens*

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob>F
Age of hemolymph	5	78.436236	15.6872	121.78 84	<0.0001
Error	24	3.091378	0.12880		
Corrected Total	29	81.527614			

Mean ± Std. Error of Protein quantity (mg/ml) of hemolymph samples

Type of hemolymph	age of hemolymph (in days)	Mean ± Std Error
1. Unparasitized hemolymph		
UP	1	2.01 ± 0.16^{aA}
UP	3	3.72 ± 0.16^{bA}
UP	5	4.94 ± 0.16^{cA}
2. Parasitized hemolymph		
P	1	1.31 ± 0.16^{aA}
P	3	4.39 ± 0.16^{bA}
P	5	5.96 ± 0.16^{cB}

UP = hemolymph from the unparasitized fifth instar *H. virescens* larvae, P = hemolymph from the parasitized fifth instar *H. virescens* larvae., 1= day 1, 3 = day 3, 5= day 5. Means within columns with different lowercase letters (a, b & c) are significantly different ($P<0.05$) within that particular type of hemolymph samples (unparasitized or parasitized) of the fifth instar *H. virescens* larvae. Means within columns with different uppercase letters (A& B) are significantly different ($P<0.05$) between the same age of hemolymph (day 1, day 3 and day 5) samples from the unparasitized and parasitized fifth instar *H. virescens* larvae ($P<0.05$).

Hemolymph protein quantification

There was a steady increase in the total protein quantity between the hemolymph samples as the age of the larvae increased in both the unparasitized and parasitized *H. virescens* larvae (Table 17). The total protein quantity was higher in the hemolymph of the parasitized larvae than in the unparasitized larvae except in day 1; UP1 had higher protein quantity than P1. Statistically, there were significant difference in protein quantities between UP1, UP3 and UP5 and also between P1, P3 and P5 ($P>0.05$). However between the unparasitized and parasitized hemolymph samples, there was significant difference only between UP5 and P5 ($P>0.05$).

Comparison of the hemolymph proteins

By visual observation, no major differences were found in the protein titer pattern between the unparasitized and parasitized fifth instar day 1, day 3 and day 5 hemolymph of *H. virescens* larvae (Fig. 7, 8, 9 a and b). There were no new proteins or missing proteins found between the same aged larval hemolymph of the unparasitized and parasitized larvae. However, the difference in spot intensity may represent either a quantitative difference in the protein (a darker spot might represent a higher quantity of protein) or a qualitative difference (presence of more than one protein in that location).

Comparison between the day 1 (UP1), day 3 (UP3), and day 5 (UP5) hemolymph protein titers of the unparasitized fifth instar* H.virescens *larvae

The intensity of protein titers in the UP1, UP3, and UP5 followed a typical pattern – low in UP1, high in UP3 and again became low in UP5. Above MW 200 kD,

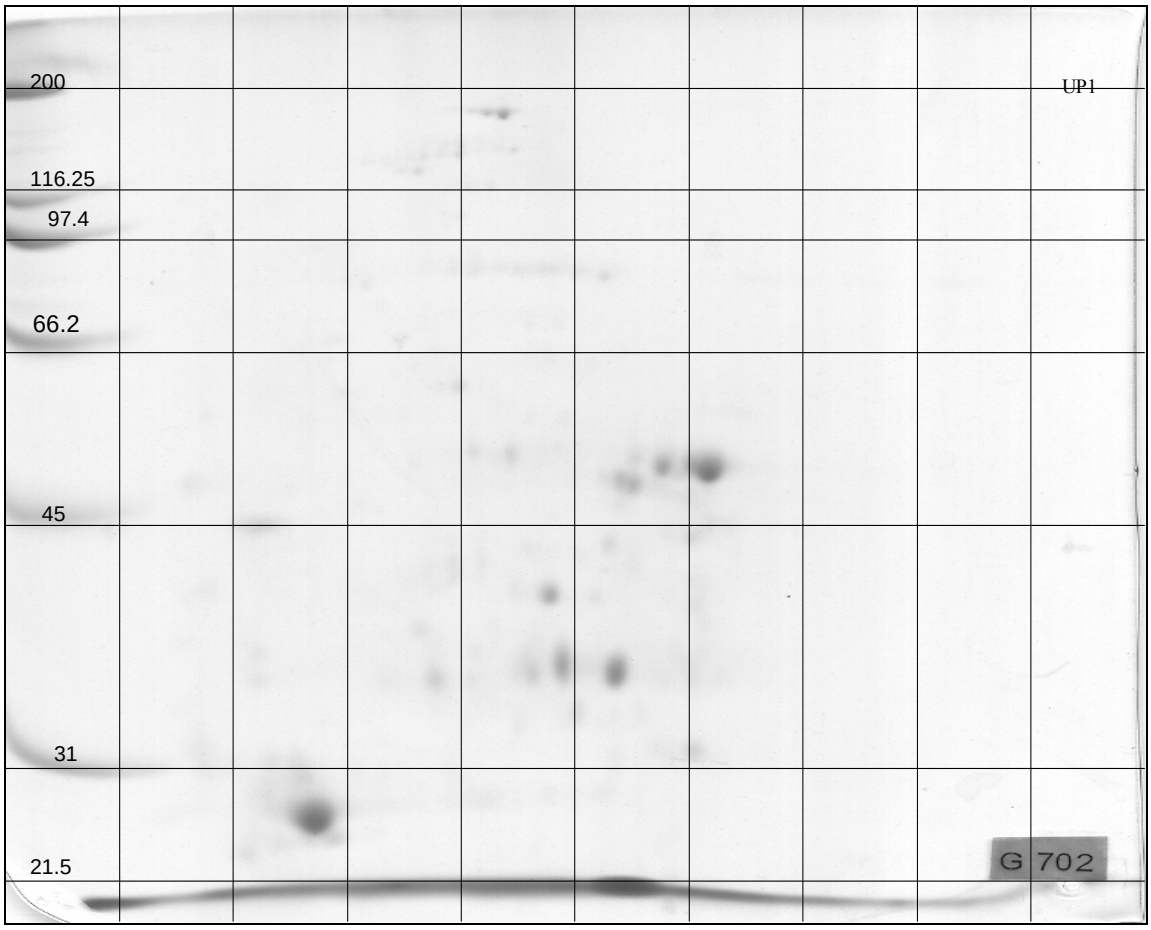

Fig. 7a. 2D gel of the first day hemolymph sample (UP1) of unparasitized fifth instar *H. virescens* larva. Molecular weights of known standard proteins are shown inside each row of column 1.

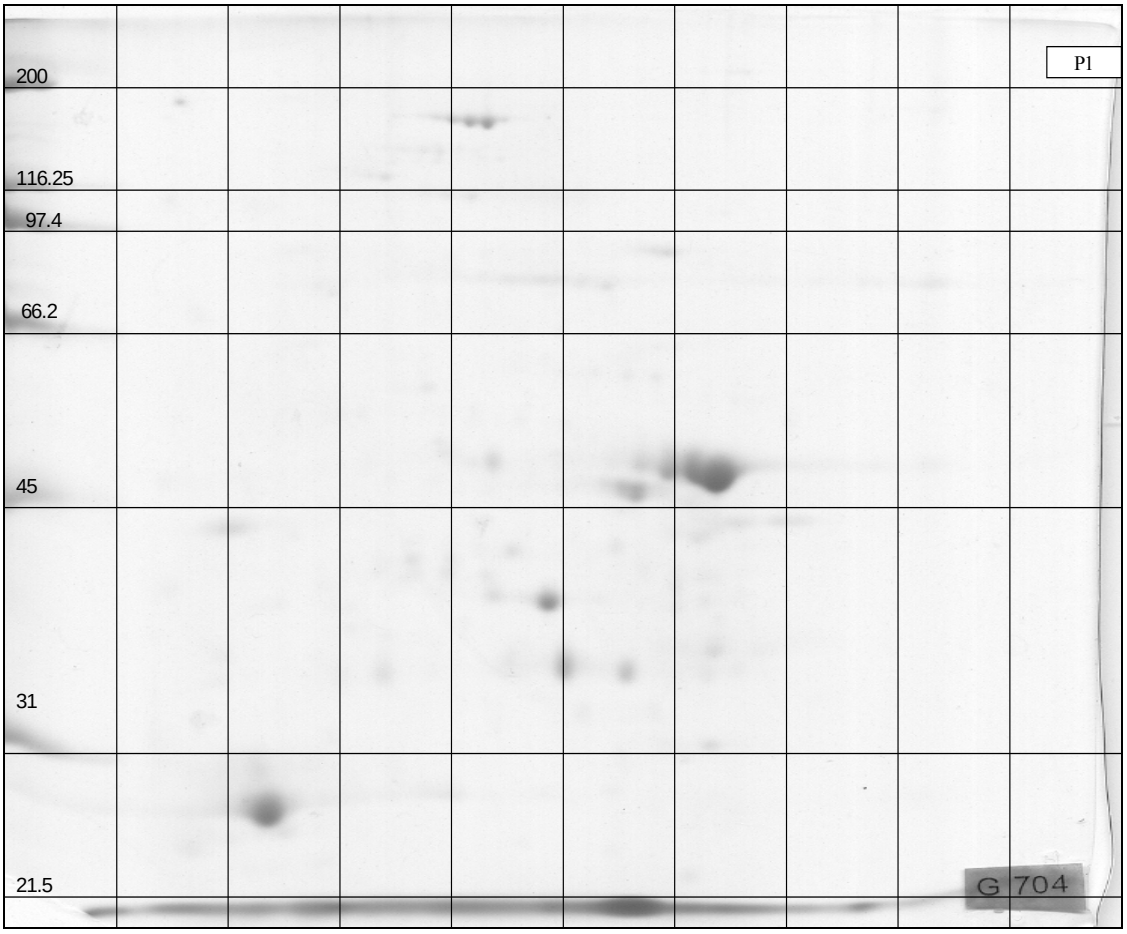

Fig. 7b. 2D gel of the first day hemolymph sample (P1) of parasitized fifth instar *H. virescens* larva. Molecular weights of known standard proteins are shown inside each row of column 1.

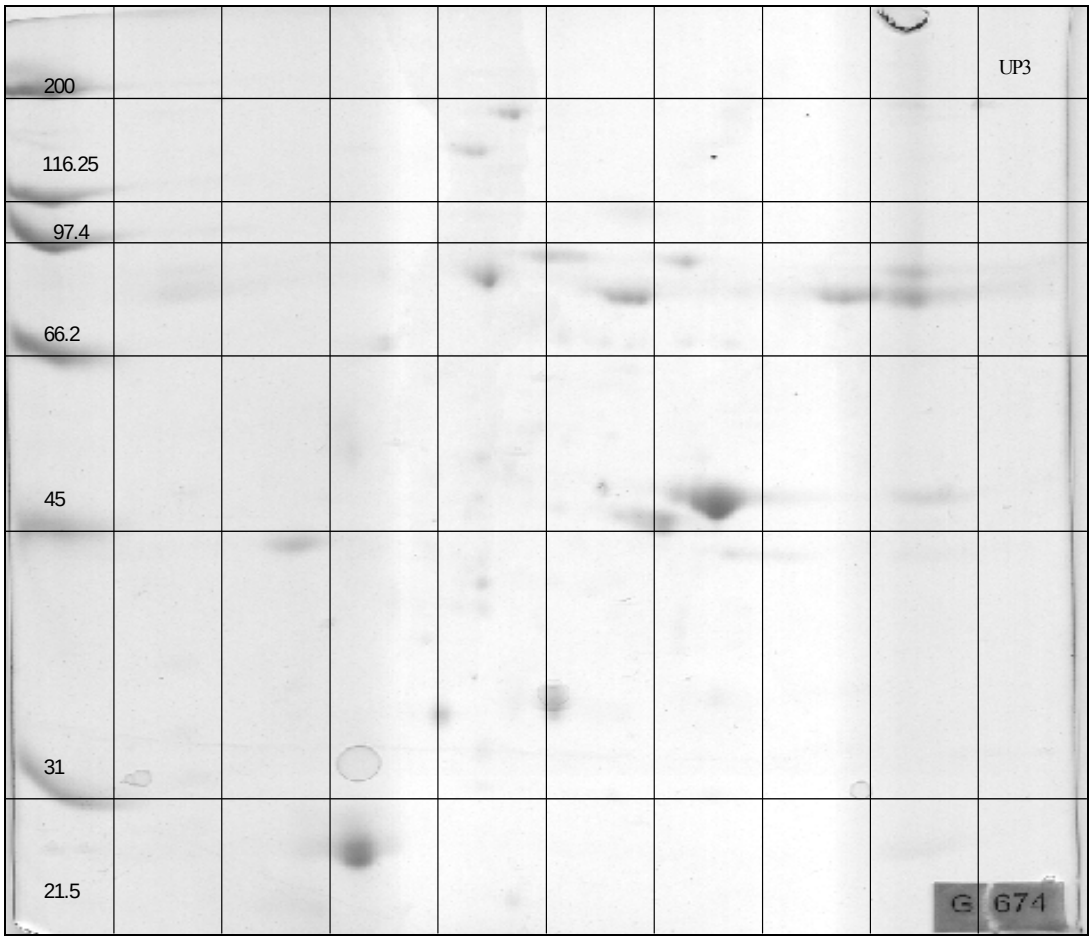

Fig. 8a. 2D gel of the third day hemolymph sample (UP3) of unparasitized fifth instar *H. virescens* larva. Molecular weights of known standard proteins are shown inside each row of column 1.

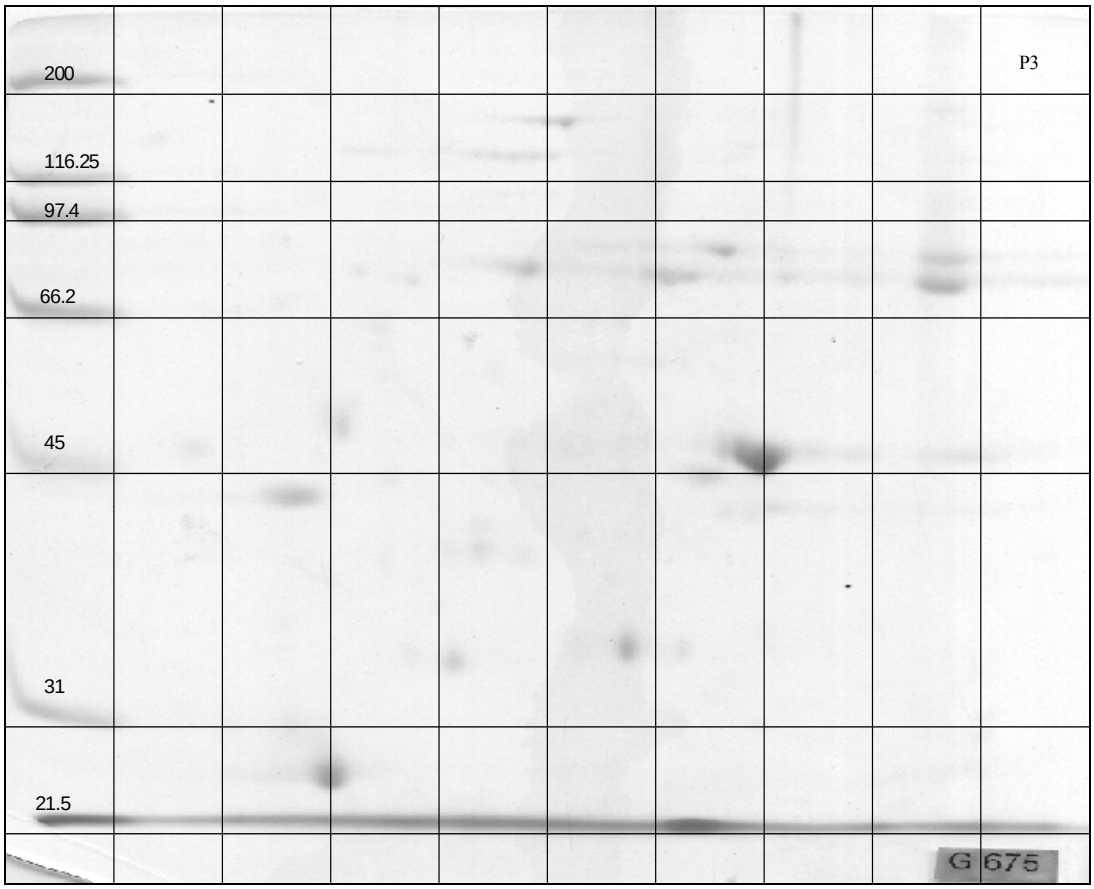

Fig. 8b. 2D gel of the third day hemolymph sample (P3) of parasitized fifth instar *H. virescens* larva. Molecular weights of known standard proteins are shown inside each row of column 1.

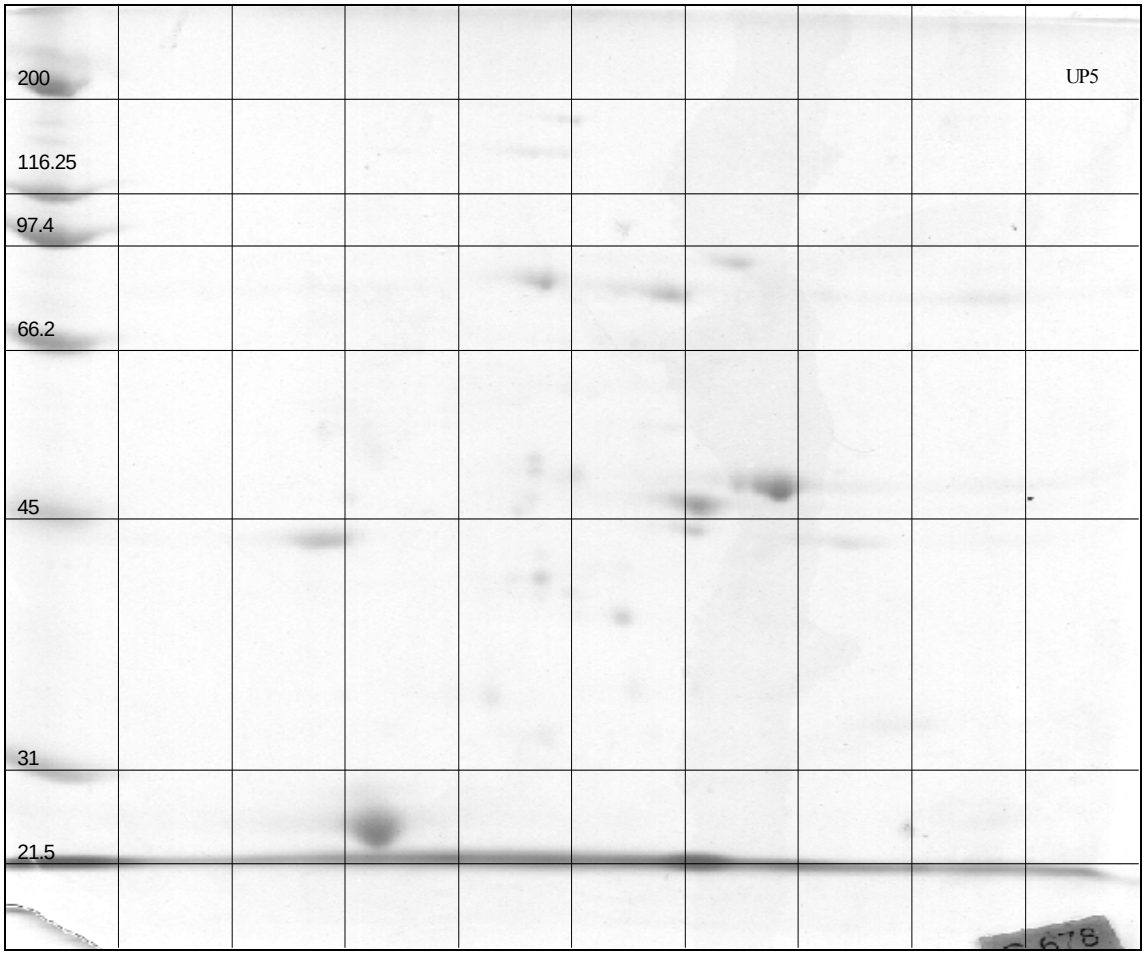

Fig. 9a. 2D gel of the fifth day hemolymph sample (UP5) of unparasitized fifth instar *H.virescens* larva. Molecular weights of known standard proteins are shown inside each row of column 1.

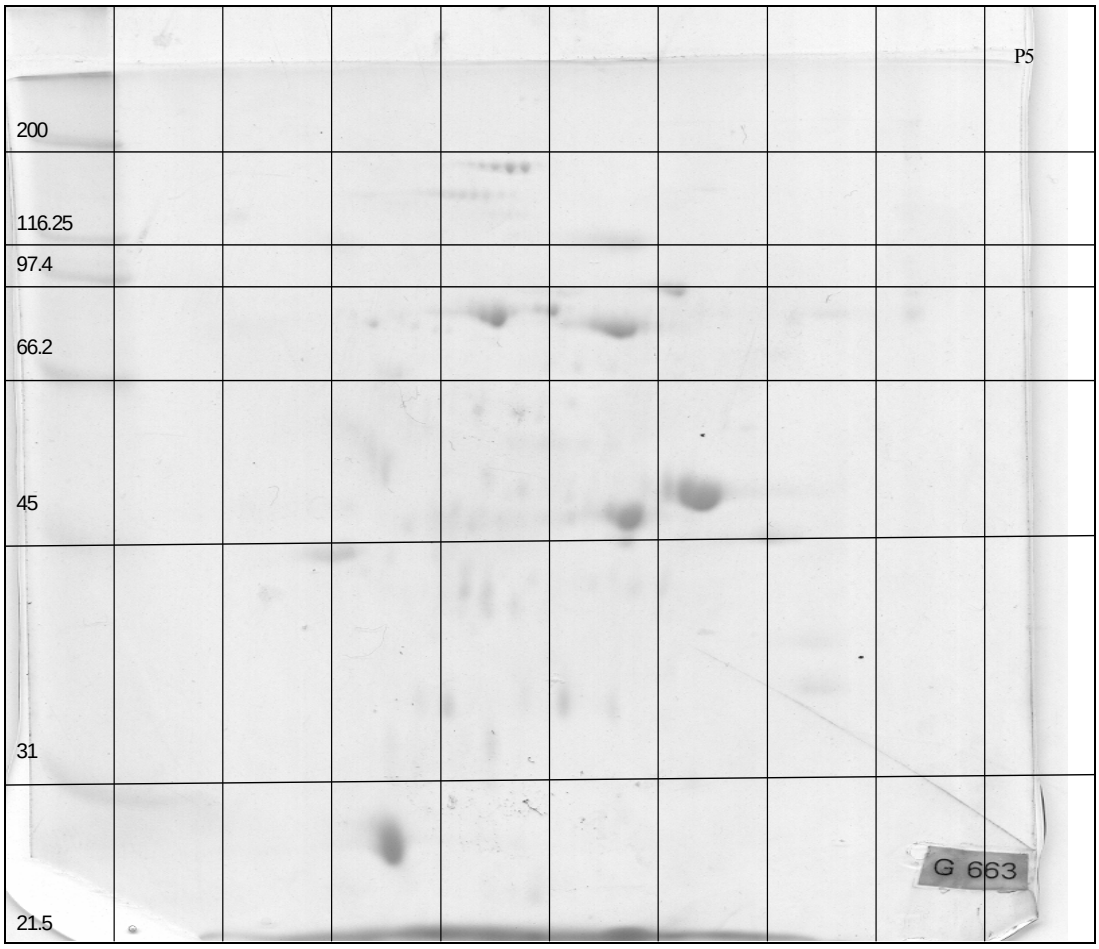

Fig. 9b. 2D gel of the fifth day hemolymph sample (P5) of parasitized fifth instar *H.virescens* larvae. Molecular weights of known standard proteins are shown inside each row of column 1.

no spots were observed in any of the gels. Between MW 200 and 116.25, in column 5, there was one spot in the UP1, 2 spots in UP3 and UP5, however, the two spots in UP3 were darker than in UP1 and UP5 (Figs. 7a, 8a and 9a). No spots were present between 116.25 and 97.4 in any of the three gels.

Between 97.4 and 66.2, there was one spot in each column 5, 6 and 7 of the day 3 and day 5, but this spot was absent in day 1 (UP1). Considering their molecular weights, these three spots may correspond to the three storage proteins of MW 74,000, 76,000, and 82,000 (Leclerc and Miller, 1990) that were found to be present in the later larval and pupal hemolymph and the pupal fat body of the tobacco budworm. The intensity of these three spots was higher in UP3 than in UP5. Likewise there was one spot in column 8, and 2 spots in column 9 in UP5, which were absent in both UP1 and UP3.

Between MW 66.2 and 45, there were 2 spots in column 6 inn UP1where as only one spot each in UP3 and none in UP5. In column 7, there was one big spot in UP1 and 2 spots in UP3 and UP5. The intensity of these spots was higher in UP3 than UP1 and UP5. Between MW 45 and 31, there were two dark spots in column 5 and one spot in column 6, whereas only one spot each in column 5 and 6 in UP3 and UP5. In UP5, these spots appeared to be lighter than in UP1 and UP3.

Between MW 31 and 21.5, there was one spot in column 3 in UP1 where as in UP3 and UP5 there was no spot in column 3, but there was one in column 4. However, the intensity of these spots appeared to be the same in all gels.

Comparison between the day1 (P1), day 3 (P3) and day 5 (P5) hemolymph protein titers of the parasitized fifth instar* H.virescens *larvae

It was observed that the protein titers of the P1, P3, and P5 followed the same pattern as in UP1, UP3 and UP5 except differences in the intensity of some of the spots (Figs. 7b, 8b, 9b). For example, the intensity of the 3 spots (representing the three storage proteins described earlier) were higher in P5 than in P3. Between MW 45 and 31, the two dark spots in column 5 and one spot in column 6 in P1 and one dark spot in column 5 and 6 in P3 were absent in P5. Instead, 6 spots in column 5 were present in P5 which were not present in either P1 or P3. These spots were extremely light which might represent the beginning of the appearance of some new parasitoid secreted proteins reported by Consoli (unpublished data) from the sixth day onwards of the parasitized fifth instar larvae. Other than these, there were no differences between P1, P3 and P5 hemolymph proteins.

Comparison of hemolymph protein titers between UP1 and P1, UP3 and P3 and, UP5 and P5

There were no significant differences in the presence or absence of spots between the unparasitized and parasitized hemolymph samples except in the fifth day samples. However the intensity of the spots were different (lighter or darker) which could be either due to the difference in the quantity of a particular protein or the presence of more than one protein in that spot (Figs. 7, 8, 9 a and b). In the fifth day parasitized hemolymph sample, between MW 45 and 31, there were six light spots in column 5 whereas only one light spot was present in unparasitized hemolymph sample.

IV DISCUSSION

This study showed that except for the initial development of the first instar larvae, these rearing media, especially the semi solidmedia improved the growth and development of the *T. nigriceps* larvae significantly. Growth was demonstrated in all the seven types of media including C (without hemolymph), however the rearing media with the parasitized hemolymph demonstrated better growth (increase in size) and development (molting to next larval instar). Among P1, P3 and P5 rearing media, P5 provided highest growth. The percentage of molting was also higher in P5. In P3 and P5, the late first instars grew well and molted to second instars and in some cases there was a second molt to the third instars. The second instar (early and late) larvae also grew well in these artificial rearing media and molted to third instars. In P5, some of the in vitro reared third instars showed signs of cocoon formation and expressed behavioral changes related to preparation of pupation. Between the semisolid and liquid rearing media, semisolid media provided better growth and development than the liquid media. However, growth of the new first instar and early first instar larvae were much less in all the media and they never molted. This may be due to the loss of some essential growth factors from the hemolymph upon collecting.

Prevention of the possible loss of labile essential growth factors is a challenge due to melanization of the host hemolymph. Melanization causes the hemolymph to turn black in color and as this change occurs the hemolymph becomes toxic to the

developing larvae, possibly due to the development of secondary products. Melanization is caused by phenyloxidase, an important enzyme found in insects. This enzyme initiates production of opsonins for phagocytosis, participates in coagulation, initiates encapsulation and mediates microbial killing (Soderhall & Smith, 1986). Phenoloxidase is involved in the formation of melanin by oxidation of phenols to quinines which are then converted to melanin by non enzymatic reactions (Soderhall & Smith, 1986). Although holding the hemolymph at 0 to 4°C slowed down melanization, it did not prevent it. Even when diluted in rearing media and when the media was brought to the rearing temperature (29±1°C), it still darkened. So inhibition was considered. Factors that affect the activity of phenoloxidase include ethylenediaminetetraacetic acid (EDTA), calcium, pH, and temperature. In an early effort to prevent melanization, I added either 0.1% or 0.5% of dithioerythritol to the artificial rearing media. Dithioerythritol is a common preservative and is non-toxic when used in low concentrations. However, dithioerythritol did not prevent melanization.

I next tried phenylthiourea which can inhibit phenyloxidase activity, but can be toxic. I injected 10 µl, 5 µl, 1µl of 2%, and 1% (w/v) phenylthiourea to the unparasitized and parasitized *H. virescens* larvae. Both the unparasitized and parasitized larvae died within 2 days whereas the control larvae injected with the same volumes of distilled water developed normally. The toxicity of phenylthiourea was of concern and so it was abandoned. I also followed the traditional way of collecting hemolymph by heating the larvae at 60-65°C for 15 min prior to

collection. This method may result in the loss of some proteins due to heating and binding to tissues. To avoid this, the hemolymph was collected in an anticoagulant buffer without prior heating of the larvae. This extracted hemolymph was heated at 62°C for 2 min to inactivate the phenyloxidases. This procedure reduced melanization and was adopted. However, even the quick heating may result in the loss of some important thermolabile factors. There are other concerns, for example, lipids and some other factors can be lost by oxidation. Also one of the concerns expressed by Pennacchio et al. (1992) and personal observations (Kuriachan and Vinson) was that the larvae reared in vitro were fragile and appeared to lack stored proteins. Therefore, I focused on higher weight factors, particularly the storage proteins. I used a 10000NMWL centrifugal filter to concentrate the hemolymph proteins so that factors below 10kD might have been lost .We can assume that these factors may be critical for the early development of the parasitoid larvae, since the early first instar larvae did not grow well in the artificial rearing media. In contrast, these factors may be less important in the growth of the late first instars and the second instars since these larvae grew and molted to the next larval instars in these same rearing media. The idea that certain low molecular factors are important for the development of early instar parasitoids would fit well with the similar concept of Vinson & Hegazi (1998) for the physiological suppression of competing larvae. A focus on these possible small molecules weight factor and early *T. nigriceps* development is an area of research that needs to be addressed.

As Pennacchio et al. (1992) reported, 100% hatching of the post germ band egg

was observed in all the tested rearing media and also in the commercial insect rearing medium, TNM-FH. None of them survived after 48 hours in any of the media that I tested; however Pennacchio et al. (1992) reported a 10% molt to second instar when the rearing media was formulated with 20% milk; but they did not survive. My initial studies were conducted with the post-germ band eggs using the Pennacchio diet (Pennacchio et al., 1992) and the Hu diet (Hu andVinson 1997) as the artificial basic medium. The eggs were explanted to the media 30 hours after parasitization (They normally hatch between 2-6 hours). In both media, 100% hatching was obtained within 2-8h after explanting; however, the first instar larvae did not show any growth and the larvae died within 48 hours after hatching. However, in the Hu diet, the larvae were more active and survival time was also longer (48 h). Then I incubated the new first instar larvae from hosts in the Hu diet (as the artificial basic medium) with different concentrations (0.1%, 0.5% and 1% of the amino acids) and finally, based on the preliminary results, chose the artificial basic medium with 1% amino acids (materials and methods for the preparation of ABM) as the best concentration for the experiment.

Nakahara et al. (1999, 2002) showed that lipophorin, one major insect hemolymph protein, is essential for in vitro development of the larval endopasrasitoid, *Venturia canescens* (Hymenoptera: Ichneumonidae) from first instar to second instar. The main function of lipophorin is transport of lipids throughout the insect hemocoel as a reusable shuttle (Shapiro et al., 1988, Blacklock and Ryan, 1994, Ryan and van der Horst, 2000). It is assumed that host lipophorin may act as a

cue for *V.canescens* development to the second larval instar and provides dietary lipids to support larval growth (Nakahara et al., 2002). In this study the lipophorin was present in the diet, isolating the lipophorin from the budworm larval hemolymph and supplementing the rearing media as a stimulant was not tried and whether it would be able to stimulate the initial development of the first instar *T. nigriceps* larvae is unknown.

Osmotic pressure can be another reason for the poor growth and mortality, particularly of the newly molted and early first instar larvae. Most of the parasitoids developed in diets with an osmotic pressure ranging from 340 to 525 m0sm, which is close to the osmolarity of the hemolymph of the host of these species (Consoli and Parra, 1999). Yet, studies on the development of chemically defined diets clearly demonstrated that the importance of dietary osmotic pressure varies between parasitoid species (Thompson and Hagen, 1999). In this study, the osmotic pressure of the host hemolymph at the first day, third day and fifth day was between 550 and 620 m0sm, whereas the osmolarity of the rearing media was between 900 and 1050 m0sm. Although the late first instar and second instar larvae grew and molted in these rearing media with the elevated osmotic pressure it may have been less suitable for the new and early first instar larvae. However, there are other factors that relate to changes in the osmotic pressure of the media. In the case of new first instar larvae, the osmotic pressure of the media increased. This would indicate that some molecules were either added or water removed. We might also predict that this increase is due to the secretion of waste products into the surrounding media as has

been shown by Edson and Vinson (1976) for *Microplitis croceipes*, another larval endoparasitoid of *Heliothis* sp. Although later instars of *T. nigriceps* larvae may secrete factors to influence the host, in early first instar larvae, the release of such molecules are more likely. The osmotic pressure of all the other media containing older larvae are decreased as might be expected as peptides, amino acids and other components of the media are absorbed and utilized in larval growth. The change would also indicate that the diets may become limiting once the nutrients are used up. In the host these may be replaced by tissue catabolism and release into the hemolymph. This possibility needs to be further investigated.

Between the liquid and the semisolid rearing media, the larval growth and molt were higher in the semisolid media, which supports the finding of Hu and Vinson (1998) in regard to *Compoletis sonorensis* reared from egg to prepupa in a semisolid media with multilayered agar and egg yolk. Although the agar fortified diet was better than the liquid diet for the late first instar and the early and late second instar larvae, these larvae consumed large quantities of agar which was used as a thickening agent. It is not clear how digestible agar is or its nutritional value. If undigested, it could influence molting, if molting is due to volume increase in the gut. If the agar is indigestible and it would be of no nutritional value, then it could have become detrimental for further nutritional intake and development as well. Evaluation of other thickening agents may be fruitful.

The pouring of the media to one side of the well, in the shape of a crescent moon and keeping the culture plate at 45° angle (slanting upwards) improved the growth of

the larvae by giving them the freedom to move in and out of the media and thereby reducing the chance of drowning. Further, it seems that the semisolid media resembled the internal milieu of the host to a certain extent, in that the first instar larvae were found to be in the liquid filled cracks of the media. In vivo, the same aged larvae are found oriented lengthwise alongside the digestive tract and fat body of the host larva, with its head forward (Lewis and Vinson, 1968, personal observation).

The average size of the newly molted second instars (2.7mm long & 0.7mm wide) was much smaller than those developed in vivo (3.67mm long and 0.9 mm wide). Likewise, the average size of the newly molted third instar larvae developed from the late first instar and early second instars were was also smaller (7mm long and 2.2mm wide) than their counterparts in vivo (9.0 mm long and 2.6mm wide). This would indicate that the intake of nutrients was less than optimal, but was capable of sustaining development. However, the average size of the third instars molted from the late second instar (8.6mm long and 2.5mm wide) was somewhat closer to those that develop in vivo (9.0 mm long and 2.6mm wide). However, the color of the third instar larvae was transparent and fragile, whereas *in vivo*, they are opaque and sturdy. The smaller size and fragility may be partially responsible for not obtaining pupation of these third instar larvae in vitro. These results would suggest that a major problem may be the concentration of nutrients available over a sustained period of time. Changing the larvae to new diet every 2 days or a comparison would substantiate this possibility.

The results of this study confirms the finding of Pennacchio et al. (1993), that there was a steady increase in the concentration of hemolymph proteins in both the unparasitized and parasitized (by *T. nigriceps*) H. *virescens* larvae from day 1of the fifth instar, and there were no significant difference in the protein concentration until the 4th day of fifth instar. Although lower than all other larval instars, I found that the protein concentration of the fifth instar day 1 unparasitized larvae was higher than that of the day 1 parasitized larvae. As the age of the larva increased, the hemolymph protein concentration also increased particularly in the parastized larvae. This increase in protein concentration showed a positive effect in the growth and development of the parasitoid. There was a significant difference in the protein concentration between the day 5 hemolymph of the unparasitized and parasitized *H. virescens* larvae. Except for tests using UP1 and P1, all the ages of the parasitoid larvae showed an improved growth in all other hemolymph containing media; the C medium (without hemolymph) was found to be the least effective. These results confirm that the presence of host hemolymph has a positive impact on the parasitoid growth and development. Further, there were little qualitative differences between the parasitized and unparasitized hemolymph, although the media with the day 3 and day 5 hemolymph (P3 and P5) were superior, possibly due to the higher protein concentration.

Although, I was successful in obtaining development from the late first instar, through the second instar to the third instar, no further development was obtained. For pupation, the larvae may need more energy or resources (protein) than they

received from the artificial diet. It has been reported that there is a significant increase in the protein concentration (78.3mg/ml) on day 7 of the fifth instar parasitized *H. virescens* larvae (Pennachio et. al 1993). This increase may be due to protein released by the host in response to the 'factors' released by the parasitoid or due to proteins produced by teratocytes, cells derived from the embryonic membrane of the parasitoid egg. Consoli & Vinson (unpublished data) detected the presence of some parasitoid secreted proteins (PSPs) of about 200kD, in the host hemolymph from day 6 of the fifth instar parasitized larvae. These parasitoid secreted proteins may serve as a trigger to manipulate the host in the final stages of its development. Furthermore, it was reported that the teratocytes play an important role in parasite nutrition (Dahlman, 1990, Dahlman and Vinson, 1993, Pennachio et al., 1992, Consoli and Parra, 1999). These cells have trophic, immunosuppressive, and secretory functions but their specific activities depend upon the life stage of the host, the time spent by the parasitoid within the host, and the individual species of parasite and host (Dahlman, 1991, Dahlman and Vinson, 1993).

Okuda et al. (1998) reported that the teratocytes of *Dinocampus coccinellae* snythesized abundant amount of a protein (TSP) which was accumulated in the teratocyte cells and the developing parasitoid larvae consume these hypertrophic teratocytes in the final stage of their development. However, in this study there was no evidence of unique 'new' abundant protein in the parasitized larval hemolymph of the day 1, day 3 and day 5 of the fifth instar, that was important for the parasitoid growth. Strand et al. (1986) suggested that teratocytes of *T. heliothidis* aided in

decomposition and necrosis of the tissues of the host, *H. virescens*, partially due to the release of lytic enzymes. Likewise, Consoli (personal communication) reported that the teratocytes released by the *T nigriceps*, helped degenerate the host tissues including the fat body which was then consumed by the third instar larvae before its egression from the host. As I explained earlier in the introduction, the third instar larva egresses from the host with the head first. While it is still attached to the host by its caudal horn, it curls and reinserts its head into the host and begins feeding in the posterior end of the host. The larva consumes all the remaining body fluids and tissues except the head capsule and the cuticle. This significant change in the parasitoid's behavior at this stage makes it an ectoparasitoid and is in air feeding on protein solids. Here it greatly increases its protein storage to be utilized for immediate cocoon formation and further development. Hence we can conclude that the consumption of the host tissues may be vital for the further development of the third instar *T. nigriceps* larvae. So, inclusion of hemolymph of the parasitized larvae from day 6 onwards and addition of host tissues in the artificial rearing media may possibly enhance further development of the parasitoid larvae. The preliminary results of my assays in P5 semisolid media with the third instar larvae, which was obtained immediately after egression and preventing consumption of the host tissues after egression, which they normally do, 50% of the larvae pupated and became adult males. However, no cocoons formed.

Since this study focused on the improvement of the artificial rearing media with the addition of host hemolymph, other factors such as availability of oxygen,

luminosity and temperature were not tested. Though the optimal conditions recorded by the previous researchers were used in this experiment, there might be some specific needs for the different larval instars. Above all, the fact that the *T. nigriceps* is a larval endoparasitoid which lives in a constantly changing milieu, as it manipulates the endocrine system of the host to fulfill their ecological and nutritional needs at a spatial and temporal basis, makes the in vitro rearing of this parasitoid more challenging. Hence, the success of this project, rearing from late first instar to third instar larvae, is promising. To conclude, older larvae responded better in the artificial rearing media than the younger ones, indicating there are other inherent factors within the host which influence larval growth that artificial conditions cannot yet be properly replicated. Focusing further studies on this direction may enable the artificial rearing of *T. nigriceps* from egg to pupation.

V SUMMARY

The addition of host hemolymph to the artificial rearing media improved the growth and development of *Toxoneuron nigriceps* larvae except in the case of the new first instar (2d) and early first instar (4d) larvae.

The new and early first instar larvae showed minimum growth in all the artificial rearing media tested, indicating that some essential growth factor(s) or trigger were missing from the media.

The late first instar larvae grew well and molted to second instar and in a few cases these in vitro reared second instar larvae molted to third instars. These larvae were alive for 9 days in the artificial rearing media.

Early second and late second instar larvae also grew well and molted to the third instars in the artificial rearing media.

Some of the in vitro reared third instar larvae showed signs of cocoon formation (evidenced by an oral secretion) and expressed behavioral changes that suggested the initiation of pupation, but pupation did not occur.

The semisolid media was found to promote better growth and molting than the liquid rearing media.

Pouring of the media to one side of the well and keeping the culture plate slanting upwards improved the growth and survival of larvae developing in the artificial rearing media.

Growth and molting in C (artificial media without hemolymph), UP1 and P1 were minimum indicating the lack of enough nutrition.

Osmotic pressure of all the media showed a significant decrease after the incubation of the larvae, except that incubated with the new first instar (2d) larvae.

There was a steady increase in the protein concentration as the age of the host larvae increased, in both the unparasitized and parasitized fifth instar larvae of *H. virescens* and the increase in the protein concentration showed a positive effect on the growth and development of the *T. nigriceps* larvae. There was significant difference in the protein concentration between the hemnolymph samples of the fifth day fith instar of unparasitized and parasitized *H. virescens* larvae.

Little or no differences in the types of protein were detected in the hemolymph of the day 1, day 3 and day 5 of the parasitized and unparasitized fifth instar *H. virescens* larvae.

The third instar larvae molted in the artificial rearing media showed no further development (cocoon formation or pupation) which indicates that better nutrition is needed.

Development of the initial stages of the first instar and the final development of the third instar larvae did not happen in the artificial rearing media which denotes the requirement of some additional factors or better nutrition other than what were present in the tested artificial media.

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VITA

I, Indira Kuriachan, was born in Palakkad, a district of Kerala State in India. I lived in Palakkad until I got my B.Sc. degree in botany in 1972. After getting married to Mr. Adattu Kuriachan, I moved to Neyveli, Tamil Nadu and became a primary school teacher. After my elder son was born, I went back to college to get my B.Ed. degree in natural science in 1981.

Later, we moved to Saudi Arabia since my husband took employment with the Saudi Arabian American Oil Company (SAUDI ARAMCO). I started working as a statistical assistant in the Community Services Department of Saudi Aramco and I became a mother for the second time when my younger son was born. In 1995, I moved to College Station to join the M.S. in urban entomology at Texas A&M University. For my M.S., my research project under the supervision of Dr. Roger Gold was to investigate the response of subterranean termites to different types of termiticides.

After graduating in 1997, I started working for Dr. S. Bradleigh Vinson as a Research Associate. I worked on fire ant projects in relation to queen dominance and its effects on food flow, reproduction, and survival of the polygynous colonies of fire ant. While working as a full time employee, I joined the Ph.D. program as a part time student in the spring of 1999.

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